A STUDY OF SOME INFLUENCES ON THE NIGROSTRIATAL DOPAMINERGIC NEURONAL PATHWAY OF THE RAT BRAIN, WITH PARTICULAR REFERENCE TO THE INFLUENCE OF 5-HYDROXYTRYPTAMINE

by

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To the memory of my father

and to my family

THE UNIVERSITY *of* EDINBURGH

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Some of the results described in this Thesis have been presented as follows:

1. Arbuthnot, G.W., Garcia-Munoz, M., Nicolaou, N.M., Tulloh, I.F. and Wright, A.K. (1976). Is the striato-nigral pathway responsible for 'feedback' control of dopamine release? Brit.J.Pharmacol, 58, 272P.

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3. Wright, A.K., Arbuthnott, G.W., Tulloh, I.F., Garcia-Munoz, M. and Nicolaou, N.M. (1977). Are the striatonigral fibres the feedback pathway? In "Psychobiology of the Striatum", 31-50, A.R. Cools, A.H.M. Lohman and J.H.L. Van den Bercken (eds.), Elsevier/North Holland Biomedical Press.

4. Arbuthnott, G.W., Mitchell, M.S., Nicolaou, N.M. and Yates, C.M. (1977). Changes in dopamine metabolism after intracranial self-stimulation in area ventralis tegmenti. J.Physiol(Lond.) 267, 36-37P.

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6. Hesketh, S.E., Nicolaou, N.M., Arbuthnott, G.W. and Wright, A.K. (1978). The effect of chronic lithium administration on dopamine metabolism in rat striatum. Psychopharmacology, 56, 163-166.

7. Vestergaard, P., Sørensen, T., Hoppe, E., Rafaelsen, O.S., Yates, C.M. and Nicolaou, N. (1978). Biogenic amine metabolites in cerebrospinal fluid of patients with affective disorders. 11th C.I.N.P. Congress, Vienna-Austria.

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The work presented in this Thesis has been composed by myself with the following exceptions:

a) The 6-hydroxydopamine lesions of the substantia nigra (Section 2) were performed by my colleagues Andrew Chiu and Daniel Uguru; the biochemical assays were performed by myself.

b) Estimation of normetanephrine (Section 4) was performed by my colleague Andrew Verth.

c) The electrolytic lesions of the raphe nuclei (Section 5) were performed by my colleague Marianella Garcia-Munoz and by myself in a joint experiment in which the post operative care, behavioural tests, histochemical and biochemical estimations were performed by myself.

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SUMMARY

In Section 1, the effects of intraperitoneal administration of the DA receptor agonist apomorphine, the DA releasing agent amphetamine and the DA receptor antagonist haloperidol on DA metabolism in the corpus striatum and the substantia nigra of the rat brain are described. The concentrations of the main DA metabolites, HVA and DOPAC, were determined in these regions at various doses and time-intervals with the aim of elucidating the mechanism of regulation of DA metabolism at the two ends of the nigrostriatal DAergic pathway. On the basis of the observed similarities and differences in the drug-induced changes of the metabolite concentrations in the DA cell body area of substantia nigra and the DA nerve terminal area of corpus striatum, the hypothesis that DA released from the dendrites of DAergic neurons in the substantia nigra acts on DA 'autoreceptors' located on the same neurons to regulate the activity of the nigrostriatal DAergic pathway was examined. Parallel to that, or alternative mechanisms were proposed to exist and regulate locally and independently the DAergic neuronal activity in the two areas.

In Section 2, the biochemical effects of lesions of the substantia nigra caused by 6-hydroxy-dopamine injected stereotaxically into this area were analysed. The primary effects of the lesion on DA concentration in the striatum were correlated with the observed changes in HVA and DOPAC in the same region. Hyperactivity

of the remaining intact neurons was suggested on the basis of the finding that lesions decreasing over 90% of striatal DA produced only 30-50% reduction of the metabolite levels. Administration of apomorphine did not alter the concentrations of HVA and DOPAC in the denervated striatum, whereas haloperidol produced marked increases in the metabolite levels on both sides, but higher percentage rise on the intact side. These findings were suggestive of a presynaptic effect of apomorphine on DA metabolism, whereas haloperidol could mainly be acting at postsynaptic receptor sites to cause an increase in DA turnover.

The effect of the lesion on the cholinergic system was assessed by measuring the activity of the enzyme choline acetyltransferase (CAT) which synthesises Ach; the effect on the GABergic system was assessed by measuring the concentration of GABA and the activity of its synthesising enzyme glutamic acid decarboxylase (GAD). The neuronal interconnections of the DAergic with the other two systems were investigated after severing selectively the nigrostriatal pathway. The lesions resulted in increased GAD and GABA as well as CAT in the ipsilateral striatum; a reduction of GAD, GABA and CAT was found in the lesioned substantia nigra. An activation of the GABergic and cholinergic neurons of the striatum seemed to result from the lesion, suggesting an inhibitory influence of the intact DAergic input on these neurons. Treatment of the animals with apomorphine or haloperidol revealed a correlation between the activity of the GABA-containing neurons in the lesioned side of the substantia nigra and the supersensitive behavioural response of this side to DA receptor stimulation. A model of the intranigral and intrastriatal

neuronal interrelations was proposed on the basis of the findings of this study.

In Section 3, the acute and the chronic treatment of rats with neuroleptic drugs were compared, in order to establish biochemical correlates of the reported tolerance which develops to some of their effects. The effects on the DA-, GABA- and Ach-containing neurons were investigated and several points regarding the neuronal interconnections in the striatum and the substantia nigra were assessed. A comparison was attempted of the biochemical findings accompanying the 6-OH-DA lesions of the substantia nigra and chronic neuroleptic treatment of rats and Parkinson's disease in humans; the similarities and differences were outlined.

In Section 4, the effects of administration of L-tryptophan, either alone or in combination with MAO inhibitors (given acutely or chronically) on the metabolism of DA and NA in the rat brain are described. The resulting marked increase in 5-HT seems to cause an increase in the turnover of the catecholamines, probably as a consequence of release by displacement caused by the 5-HT that spills over their neurons. On the basis of comparison of these effects with those of amphetamine, it was concluded that L-tryptophan plus a MAO inhibitor may act like the catecholamine-releasing agent to cause the reported beneficial effects in manic-depressive illness.

In Section 5, the influence of the 5-HT system on the nigrostriatal DAergic pathway was examined, using a different approach than the one employed in Section 4. Selective asymmetric electrolytic lesions of the median raphe (MR) and the dorsal raphe (DR) nuclei of the rat brain were made. A combination of histological, biochemical

and behavioural studies were employed and the results suggested that the two nuclei project to different sites of the nigro-striatal DAergic pathway and, therefore, influence its function in a different manner. It was concluded that the MR nucleus unilaterally innervates the striatum, whereas the DR nucleus unilaterally innervates the substantia nigra. The behavioural studies suggested and the biochemical data demonstrated convincingly that these innervations are inhibitory on the target DAergic neurons, since removal of either of these serotonergic projections resulted in an increase of DA turnover in the respective terminal area. A rat turning model was demonstrated following asymmetric raphe lesions. Thus, lesions of the MR resulted in turning contralateral to the lesion upon the administration of apomorphine or amphetamine; lesions of the DR resulted in ipsilateral turning upon the administration of the same drugs. Furthermore, administration of the 5-HT receptor agonist 5-methoxy-N,N-dimethyltryptamine caused turning in opposite directions than apomorphine or amphetamine with both types of lesion. Combinations of drugs thought to act specifically either on the DAergic or on the serotonergic system were given and their effect on turning behaviour assessed. A model was proposed incorporating the serotonergic afferents to the substantia nigra and the striatum, together with the other neuronal connections which are believed to exert an influence on the function of the nigro-striatal DAergic pathway.

In the Appendix, the methodology of estimation of DA metabolites (HVA and DOPAC) in brain tissue is described. The original gas liquid chromatographic method of Pearson and Sharman was modified, to improve its recovery, decrease its complexity and

increase its specificity. The modified assay procedure enabled the estimation of HVA and DOPAC in small discrete areas of the rat brain.

ABBREVIATIONS

AC	aqueductus cerebri
Ach	acetylcholine
b.p.	boiling point
CAT	choline acetyltransferase
CC	crus cerebri
CS	corpus striatum, striatum
c.p.m.	counts per minute
CSF	cerebrospinal fluid
DA	dopamine
d-β-h (DA-β-h)	dopamine-β-hydroxylase
DHPG	3,4-dihydroxyphenylethylene glycol
DOPAC	3,4-dihydroxyphenylacetic acid
d.p.m.	disintegrations per minute
dr or DR	dorsal raphe
ECD	Electron Capture Detector
EDTA	ethylene diamine tetra-acetic acid, disodium salt
EPS	extrapyramidal side effects
FLM	fasciculus longitudinalis medialis
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
glc	gas liquid chromatography
h	hour
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
5-HIAA	5-hydroxyindoleacidic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
HVA	homovanillic acid
i.p.	intraperitoneally
LM	lemniscus medialis
μm	micron (10^{-6}m)
MAO	monoamine oxidase
5-MDT	5-methoxy-N,N-dimethyltryptamine
MHPG	3-methoxy-4-hydroxyphenylethylene glycol
mr or MR	median raphe
NA	noradrenaline
n.s.	not significant
p	probability
p.c.	pars compacta
PCPA	p-chlorophenylalanine
PCS	pedunculus cerebellaris superior
POPOP	1,4-di-[2-(4-methyl-5-phenyloxazolyl)-benzene]
PPO	2,5-diphenyloxazole
p.r.	pars reticulata
R.F. or FOR	formatio reticularis (reticular formation)
s.d.	standard deviation
s.e.m.	standard error of the mean
SN	substantia nigra
TFA	trifluoracetic anhydride
VMA	vanillylmandelic acid
Vs	versus

SECTION 1

DOPAMINE METABOLISM IN CORPUS STRIATUM, SUBSTANTIA NIGRA AND OTHER RAT BRAIN REGIONS

1.1 INTRODUCTION

1.1.1 Distribution of dopamine in brain

Dopamine (DA) has been biochemically demonstrated in various areas of the brain (1,2) and this provided evidence for a possible DAergic (that is, DA-containing) system in the brain. The Falck and Hillarp histochemical technique provided the first evidence of cellular localisation of DA (3,4). Subsequent work described the detailed localisation of the DA cell bodies and nerve terminals (5,6).

The well known nigro-striatal DAergic (that is, DA-containing) pathway originates from the area pars compacta of the substantia nigra (cell group A9 according to Dahlström and Fuxe) (5), and from the adjacent ventrolateral mesencephalic reticular formation (cell group A8) (7,8). The DA-containing fibres ascend in the area ventralis tegmenti, enter into the medial forebrain bundle, pass into the internal capsule in mid-hypothalamus, and reach the striatum (nucleus caudatus-putamen) through the globus pallidus (8).

The mesolimbic DA system originates from the A10 cell bodies, located medial to the pars compacta, surrounding the nucleus interpeduncularis and mixed with the occulomotor nerve fibres. The axons ascend more medially than the nigrostriatal fibres and terminate in the nucleus accumbens, olfactory tubercle and the septal nuclei (8,9).

The tubero-infundibular DA neuronal system originates mainly from the cell bodies in the nucleus arcuatus (cell group A12) and the ventral part of the anterior periventricular hypothalamic nucleus and innervates the external layer of the median eminence (10). A number of catecholamine-containing nerve cells (probably DA-containing) have also been found throughout most of the rostral periventricular hypothalamic area (11),

named A14. A retina DA-containing neuronal system has also been demonstrated (12), and by the use of a variety of methods DA-containing nerve terminals have been observed in the cerebral cortex (13,14) as well as the frontal and anterior limbic cortices of the rat brain (9).

Biochemical estimations of regional DA concentrations and tyrosine hydroxylase activities (15) provided further evidence concerning the distribution of DA in the rat brain.

A DA-sensitive adenylate cyclase, thought to be associated with the DA receptors (16) has been localised in and isolated from various parts of the rat brain, such as the corpus striatum (17), substantia nigra (18), cerebral cortex (19), nucleus accumbens and olfactory tubercle (20).

1.1.2 Dopamine metabolism

DA in the rat brain is metabolised by the action of the enzymes monoamine oxidase (MAO), catechol-o-methyl transferase (COMT) and indirectly aldehyde dehydrogenase (ADH) (21). 3,4-Dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine are considered as the three main products of DA degradation (21). DOPAC is only partially O-methylated to HVA, whereas the formation of HVA through methoxytyramine is unlikely (22). DOPAC turnover probably reflects DA turnover (22), though HVA has been proposed for the same role (23). The presence of both free and conjugated with sulphate or glucuronide HVA and DOPAC in areas of the rat brain, such as the striatum, the olfactory tubercle and the frontal cortex has been demonstrated (24).

Roth et al. (25) have suggested that short term changes in brain levels of DOPAC provide a useful index of alterations in the functional activity of central DAergic neurons, since drugs which increase impulse

flow in the nigrostriatal or mesolimbic DA neurons increase DOPAC levels in the rat striatum and olfactory tubercle, and drugs which reduce impulse flow lead to a reduction in DOPAC. According to this model, during periods of increased impulse flow when more transmitter is utilised, synthesis should be accelerated, and during periods of decreased impulse flow transmitter synthesis should be retarded as a result of decreased utilisation and a build up of transmitter. This is consistent with results obtained in both peripheral (26) and central (27) noradrenergic neurons, where increases and decreases in neuronal activity appear to be well correlated with increases or decreases in noradrenaline metabolism and turnover. Recent studies have shown that central dopaminergic neurons behave in a fashion very similar to both peripheral and central noradrenergic neurons in their response to increases in impulse flow (25).

DA metabolism can be studied in vivo by measuring concentrations of DA and its major metabolites, HVA and DOPAC. Distinction between effects of treatments on turnover or on release of DA can be made on the basis of measurements of the concentration of DA. Release and uptake processes could be distinguished on the basis of the measured changes in the concentrations of the metabolites, and of the assumption that DOPAC is formed intraneuronally and HVA extraneuronally (38,39,40). However, recent investigations by Korf et al. (33) suggest that DA released from DAergic neurons is predominantly deaminated extraneuronally to form DOPAC, and approximately half of the deaminated metabolite is then methylated to form HVA. Other methods of studying DA metabolism and turnover in vivo include the administration of a labelled precursor and measurement of the rate of disappearance of the newly synthesised amine by estimating the concentration of the labelled metabolites. However, such methods suffer from the fact that what is measured are the metabolites of exogenous DA.

1.1.3 Nigrostriatal dopaminergic pathway

The association of DA with synaptic transmission in the nigrostriatal pathway is well documented. Electrical stimulation in the region of the substantia nigra causes release of DA (28,29) as well as electrical responses in the corpus striatum (30). Bjorklund and Lindvall (31) and Hajdu et al.(32) have demonstrated histologically the presence of DA not only in the cell bodies, the nerve terminals and the axons, but also in the dendritic processes which project abundantly into the pars reticulata of the substantia nigra. The similarity of the storage and uptake properties of the nigral dendrites to those of the axons and the cell bodies might suggest that DA fulfills a role as a transmitter in dendro-dendritic synapses (31).

There are few reports on the influence of drugs on DA metabolism in the substantia nigra. Korf et al.(33) obtained evidence for DA release in the substantia nigra following electrical stimulation of the medial forebrain bundle. Geffen et al.(34) observed ^{3}H-DA release after potassium stimulation of substantia nigra (mainly pars reticulata) slices prelabelled with ^{3}H-DA. Javoy et al.(35) reported an increased rate of synthesis of ^{3}H-DA from ^{3}H-tyrosine in both the substantia nigra and the corpus striatum 3 hours after treating the animals with the neuroleptic drug thioproperazine. Lienhart et al.(36) demonstrated increased DA-derived fluorescence of the cell bodies of the substantia nigra, 10 min after morphine treatment or after cold stress. Perkcic and Walters (37) demonstrated a different response of the DA cell bodies in the substantia nigra and the axon terminals in the corpus striatum following the inhibition of impulse flow by the administration of γ-butyrolactone, i.e. an increase of DA synthesis in the axon terminals

but no increase in the nigral and ventral tegmental areas.

1.1.4 Purpose of the present study

There appears to exist a close relationship between functional activity of DAergic neurons and the concentrations of both HVA and DOPAC (33). However, although the regional distribution of DA and tyrosine hydroxylase have been extensively studied, methodological problems have hindered a study of the regional distribution of HVA and DOPAC. Development of more sensitive techniques than the fluorimetric methods facilitated the measurement of these metabolites in areas of the rat brain not particularly rich in DA (e.g. 41,42). In the present study, measurements of HVA and DOPAC concentrations in various areas of the rat brain have been carried out, using the modified sensitive gas-chromatographic method of Pearson and Sharman (41, Appendix of present Thesis). Particular emphasis was placed on the estimation of the metabolite levels in corpus striatum and substantia nigra. A comparison was attempted of the changes induced by drug treatments in both structures in order to clarify the similarities and differences between the cell bodies and the nerve terminals in relation to DA metabolism. This would give some insight into the function of the physiologically important nigrostriatal DAergic pathway and help elucidate a possible regulatory system functioning in connection with it.

The DA receptor stimulant apomorphine, the DA-releasing agent amphetamine and the DA receptor blocker haloperidol were used as tools for the present study. Apomorphine is thought to directly stimulate DA receptors in brain, thus leading to a feedback-mediated reduction of DA synthesis and utilisation (63). Amphetamine has, possibly, no direct action on DA receptors at physiological doses, but it is

believed to act by releasing DA from its presynaptic storage sites (43). Haloperidol is a drug that blocks the apomorphine- or amphetamine-induced stimulation of DA receptors in brain. It is known to produce an increase in DA turnover, thought to result from a secondary activation of the enzyme tyrosine hydroxylase following a primary blockade of DA receptors, probably at postsynaptic sites (44). Various other modes of action have, however, been proposed to explain the effect of haloperidol on DA turnover (see Review by Wright et al., 45).

1.2 MATERIALS AND METHODS

1.2.1 Animals and dissection

Male albino Wistar rats, 200–300 g were used. The animals were killed by a blow on the head and decapitation, after which the brains were quickly removed and transferred to a beaker containing chilled saline solution (0.9% NaCl). The brains were then placed on a glass-plate kept on ice and the various brain areas were dissected out and placed immediately into liquid nitrogen. The areas olfactory tubercle, hypothalamus and cerebellum, were first removed. Two transversal cuts were then made with a razor blade, one cut just rostral to the cerebellum and the other one 2mm rostral to the caudal end of the hypothalamus (Fig. 1.1). A part of the mesencephalic tissue was then dissected, as shown in the Figure; this included the substantia nigra and the ventral tegmental area. The easily recognised hippocampus was also dissected from the same section. Another coronal cut was made 2mm rostral to the commencement of the optic chiasma. The corpus striatum was then dissected from the two pieces (2 and 3 in the Figure) and the septum, lying between the two sides of the striatum was pinched out. Part of the cerebral cortex of the area 2 was also dissected. Area 3 was cut perpendicularly to the previous cut at the level of the anterior commisure and the small region of the nucleus accumbens was dissected according to Horn et al. (20). A sample of part of the frontal cortex lying at the rostral part of the brain was also obtained. Care was always taken during the dissection of the striatum to distinguish the caudateputamen (neostriatum, striatum) from the globus pallidus

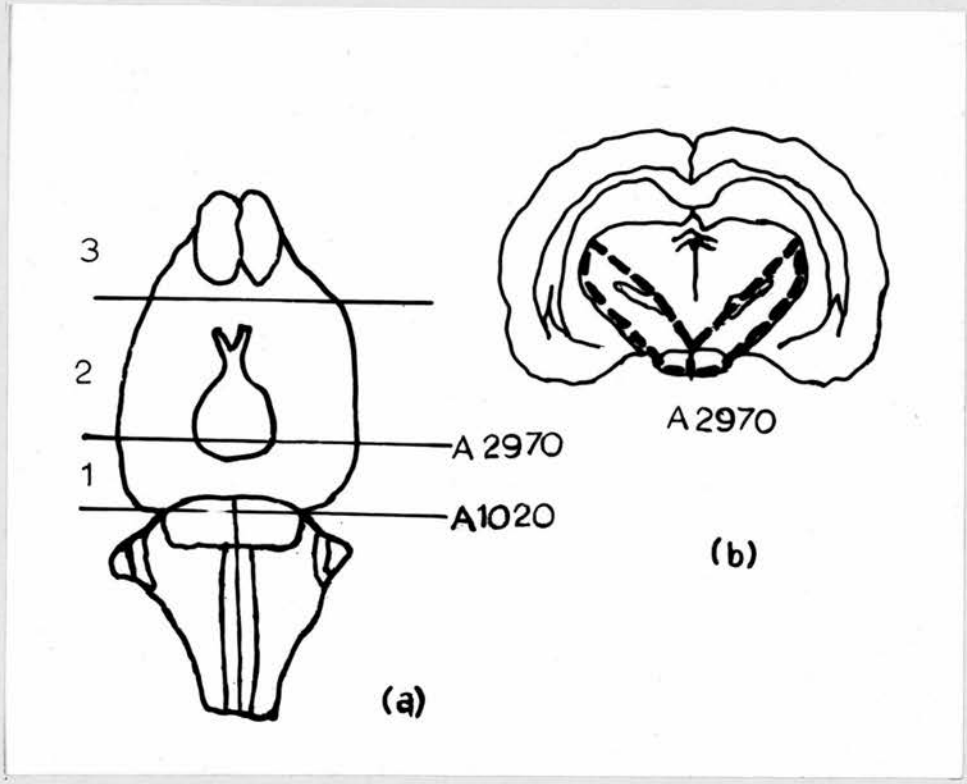


Fig. 1.1

A diagram of the rat brain illustrating (a) the levels of coronal sections used in the biochemical analyses, and (b) the substantia nigra, dissected from the tissue between the levels A2970 and A1020 of König and Klippel (140) (area indicated by the dashed lines).

(paleostriatum, pallidum).

Quoted estimates for the concentrations of HVA and DOPAC in the corpus striatum, the substantia nigra, the nucleus accumbens, the olfactory tubercle, the frontal cortex, the cerebral cortex, the hippocampus and the hypothalamus are each the mean of the bilateral parts of the dissected tissues pooled together. About 200 mg of the cerebral cortex, the cerebellum and the frontal cortex were dissected out and analysed.

1.2.2 Drug treatments

DL-Amphetamine sulphate (Sigma) and apomorphine hydrochloride (Macfarlane & Smith) were administered intraperitoneally dissolved in saline (0.9% NaCl) solution. The doses were calculated as free base. Haloperidol was used as a commercially available solution (Serenase, Jansen). Dosage schedules are given in the RESULTS.

1.2.3 Statistics

Differences between control and experimental values were analysed using Student's t test for unpaired samples (two tailed). A p value of less than 0.05 was considered significant.

1.2.4 Determination of HVA and DOPAC in brain samples

The concentrations of HVA and DOPAC in brain regions were measured with the modified and improved gas chromatographic method of Pearson and Sharman (41) as described in the Appendix. The original method was used for the assay of the two metabolites in the whole brain of rats, as described also in the Appendix.

1.3 RESULTS

1.3.1 Regional concentrations of HVA and DOPAC

The HVA and DOPAC levels in rat whole brain (including cerebellum) were 0.153 ± 0.010 μg/g (n = 8) and 0.186 ± 0.016 μg/g (n = 8), respectively, as shown in Table 1.1. The ratio HVA/DOPAC is 0.82.

The estimates of the concentrations of HVA and DOPAC in several regions of the rat brain are shown in Table 1.1. The ratio of HVA to DOPAC varies between 0.49 (nucleus accumbens) and 1.26 (frontal cortex). The highest HVA and DOPAC concentrations were found in the olfactory tubercles, nucleus accumbens, substantia nigra and corpus striatum. Other areas moderately rich in these metabolites were the hypothalamus and the hippocampus. Areas such as the cerebral cortex and the frontal cortex were also found to contain measurable amounts of the two metabolites. The cerebellum had the lowest concentrations but in sufficient amounts to be measured with the glc method.

The weights of wet tissue analysed are shown in Table 1.1. The weights for both sides of the substantia nigra, hypothalamus and hippocampus are given.

1.3.2 Effect of drug treatments

The drug haloperidol, believed to be a DA receptor blocker, given in various doses and according to different time schedules, caused apparent changes in the concentrations of HVA and DOPAC in corpus

Table 1.1

Whole rat brain and regional concentrations of HVA and DOPAC

Brain Area	Wet weight (mg)	HVA µg/g wet weight	DOPAC µg/g wet weight	HVA/DOPAC
Whole brain	1830 ± 64(32)	0.15 ± 0.10 (8)	0.19 ± 0.02 (8)	0.82
Substantia nigra**	12.18 ± 2.52(28)	1.50 ± 0.25(25)	1.22 ± 0.27(26)	1.23
Cerebral cortex		0.12 ± 0.05 (9)	0.11 ± 0.03 (9)	1.09
Hypothalamus**	26.3 ± 2.4 (6)	0.20 ± 0.04 (6)	0.31 ± 0.04 (6)	0.66
Frontal cortex		0.18 ± 0.02(21)	0.14 ± 0.02(21)	1.26
Hippocampus**	84.5 ± 12.3 (9)	0.23 ± 0.03 (7)	0.24 ± 0.02 (8)	0.96
Olfactory tubercle*	18.5 ± 3.4(27)	0.76 ± 0.16(16)	1.41 ± 0.20(18)	0.54
Nucleus accumbens*	21.6 ± 2.7 (43)	0.69 ± 0.18(43)	1.40 ± 0.22(43)	0.49
Cerebellum		0.05 ± 0.01 (5)	0.05 ± 0.01 (5)	0.96
Corpus striatum*	37.4 ± 4.1(13)	1.14 ± 0.14(30)	1.18 ± 0.14(32)	0.97

The concentrations of HVA and DOPAC were measured with the sensitive gas chromatographic procedure described in the Appendix.

The weight of dissected tissue samples is quoted for some regions (* one side; ** both sides) whereas for cerebral cortex, frontal cortex and cerebellum approximately 200 mg of tissue was used.

Figures represent means ± s.d.; the number of animals is in parentheses.

striatum and substantia nigra, as shown in Fig. 1.2 and 1.3. Control animals were treated with 0.5 ml saline solution and immediately killed.

Haloperidol, given at doses 0.4-10 mg/kg i.p. caused a dose related increase in HVA and DOPAC in the corpus striatum, but showed a plateau at doses over 1 mg/kg, especially apparent in DOPAC levels (Fig. 1.2). The concentrations of both metabolites were significantly higher than controls with all doses of haloperidol used, but the difference between the concentrations after 1 mg/kg and after 2 mg/kg was not statistically significant. A higher dose (10 mg/kg i.p.) induced a further rise in the metabolite concentrations, which were significantly higher than the concentrations after 1 mg/kg. A dose of 1 mg/kg i.p. caused a significant increase in HVA and DOPAC between 30 min and 3 hours after the injection, with a maximum effect between 1 and 2 hours (Fig. 1.3). Thereafter the DOPAC concentration showed a decline and a return to normal levels after 5 hours, whereas the HVA concentration was still significantly higher than the control at this time.

A different dose and time-effect response with regard to HVA and DOPAC was produced in the substantia nigra (Fig. 1.2 and 1.3). Doses of 0.4 and 1 mg/kg caused a significant rise in the concentrations of both metabolites 1 hour after injection, but higher doses of 2 mg/kg produced a lower percentage rise than the doses of 0.4 and 1 mg/kg, although these concentrations were still higher than the controls. The concentration of HVA was not significantly higher than control when 5 or 10 mg/kg were injected 1 hour before decapitation of the rats, whereas the DOPAC concentration was higher than controls with both doses, but significantly lower than the maximum concentration resulting from a dose of 0.4 mg/kg. The time course of the effects of

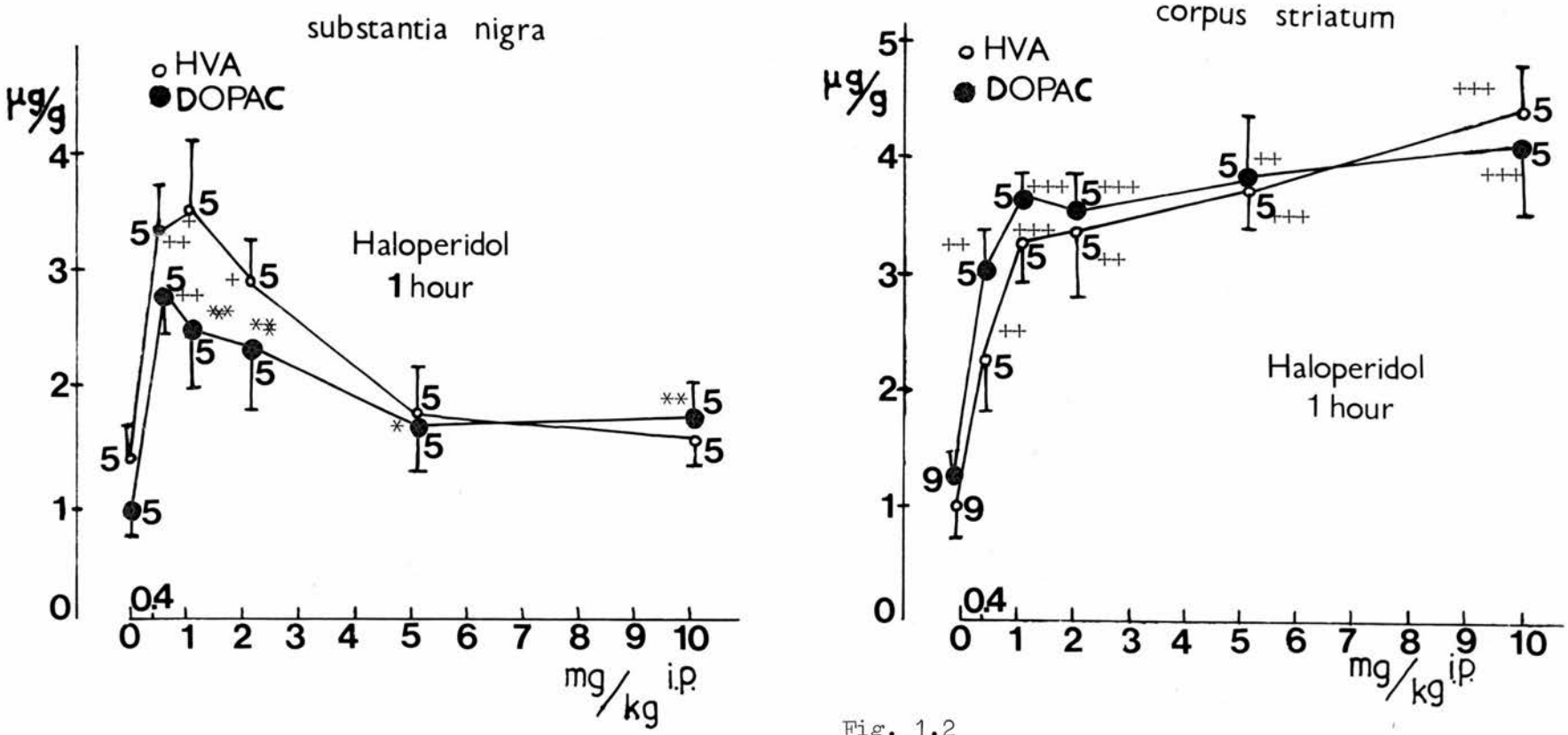


Fig. 1.2

Effect of varying doses of haloperidol on HVA and DOPAC in the substantia nigra and the corpus striatum.

All doses were given i.p. 1 hour before killing. Vertical bars represent s.d. for the indicated number of animals in each group.

Statistical significance (Student's t test, two-tailed, compared to control)

*	$p < 0.05$	+	$p < 0.005$
**	$p < 0.025$	++	$p < 0.0025$
***	$p < 0.01$	+++	$p < 0.0005$

1 mg/kg haloperidol i.p. on the concentrations of HVA and DOPAC in the substantia nigra was different from that in the corpus striatum (Fig. 1.3). A significant decrease in both metabolite concentrations 30 min after injection (to 70% of control), was followed by a significant increase to 251% for HVA and 258% for DOPAC 1 hour after injection of this dose. This peak in the time-effect curve was followed by a fall to 136% of control for HVA and 122% of control for DOPAC 2 hours after injection and then to 120% and 76% respectively, 3 hours after injection. The concentration of HVA was still significantly higher than controls 2 hours after injection, whereas DOPAC was not different than control levels. 3 and 5 hours after administration of 1 mg/kg haloperidol, the HVA concentrations were not significantly different than controls, whereas the DOPAC concentrations were significantly lower than controls (70% at 3 and 76% at 5 hours).

Figure 1.4 summarises the effects of apomorphine (a DA receptor stimulant) on HVA and DOPAC in the corpus striatum and the substantia nigra. The concentrations of HVA and DOPAC in the corpus striatum at 10 and 20 min after the i.p. injection of 1 mg/kg apomorphine were significantly reduced to 72% and 81% (HVA) and 62% and 60% (DOPAC) of the control levels, respectively. At 20 min, the reduction following a 2 mg/kg dose was not greater than that after a 1 mg/kg dose. In the substantia nigra on the contrary, at 10 min after a dose of 1 mg/kg was given, there was a significant rise in the concentrations of both HVA and DOPAC to 125% and 129% respectively, but at 20 min after this dose there was a significant decrease in both metabolites to about the same extent as in the corpus striatum (77% and 68%). In both brain areas, a dose of 1 mg/kg of apomorphine caused the maximum effect and a dose of 2 mg/kg did not produce a greater response.

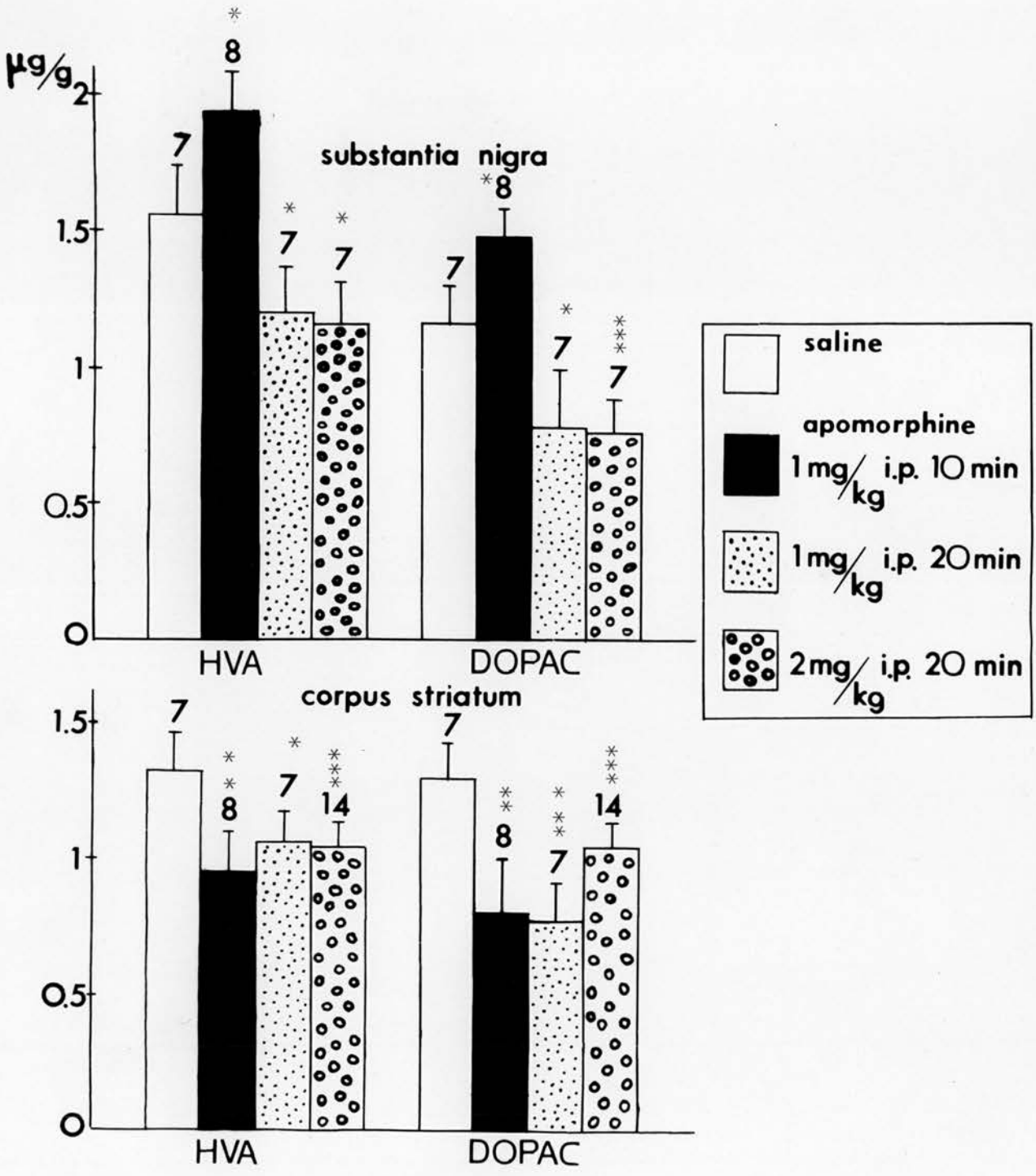


Fig. 1.4

Effect of apomorphine on the concentrations of HVA and DOPAC in the substantia nigra and the corpus striatum

Vertical bars represent s.d. for the number of observations indicated.

Statistical significance (Student's t test, two-tailed):

* $p < 0.01$
** $p < 0.005$
*** $p < 0.0025$

DL-Amphetamine (Table 1.2), in a dose of 5 mg/kg i.p. 1 hour before killing caused a significant reduction in both HVA and DOPAC in the substantia nigra ($p < 0.01$ and $p < 0.05$, respectively). In the corpus striatum, the DOPAC concentration was decreased significantly, ($p < 0.01$), whereas HVA had not changed.

Table 1.2

Effect of amphetamine on dopamine metabolite concentrations in corpus striatum and substantia nigra

	Corpus striatum		substantia nigra	
Treatment	HVA	DOPAC	HVA	DOPAC
Saline	0.96 ± 0.17(8)	1.10 ± 0.16(8)	1.79 ± 0.22(8)	1.18 ± 0.21(8)
DL-Amphetamine	1.10 ± 0.16(8)	0.62 ± 0.19(8)**	1.23 ± 0.20(8)**	0.78 ± 0.18(8)*

Rats were injected with DL-amphetamine (5mg/kg i.p.), sacrificed 1 hour later and the concentrations of HVA and DOPAC measured in corpus striatum and substantia nigra.

Control animals received saline solution.

Results are expressed as $\mu g/g$ tissue, wet weight.

Values represent means ± s.d.; the number of animals is in parentheses.

Significance of differences from control values:* $p < 0.05$; ** $p < 0.01$.

1.4 DISCUSSION

1.4.1 Tissue levels

By comparison with earlier measurements of the concentrations of HVA and DOPAC in rat whole brain and in brain areas the values obtained in this study were in good agreement, as shown in Table 1.3. The different dissection procedure, the strain of rats used and the different recoveries of the various methods may account for the differences between the reported estimates of the two metabolites. With regard to areas not clearly defined, such as the nucleus accumbens, frontal cortex, substantia nigra, the difference between the reported values may result mainly from the dissection procedure used in each study. The low recoveries of the fluorimetric procedures used for some assays (47) may explain the generally low values obtained, compared to the results of studies using the present and other gas chromatographic techniques.

The ratios HVA/DOPAC seem to suggest that DOPAC is the main DA metabolite in certain areas of the brain, such as the nucleus accumbens and the olfactory tubercle (ratios 0.49 and 0.54 respectively) and probably the hypothalamus (ratio 0.66) in agreement with previous reports (33,42). Several other areas, however, such as the striatum, the cerebral cortex, the hippocampus and the cerebellum had ratios of about 1. Furthermore, areas such as the substantia nigra and the frontal cortex had ratios of 1.23 and 1.26 respectively. Although the functional significance of these variations is not known, HVA could be equally suggested as the main metabolite reflecting DA metabolism in certain areas with ratio HVA/DOPAC greater than 1, as it has been proposed by

Table 1.3

Comparison of reported estimates of HVA and DOPAC concentrations in the rat brain with those of the present study

Brain Area	HVA (µg/g)	DOPAC (µg/g)	Procedure	Reference
Whole brain	0.153 ± 0.010 (8)	0.186 ± 0.016 (8)	*	Present study
	0.074 ± 0.003(13)	0.064 ± 0.007(13)	+	42
Corpus striatum	1.14 ± 0.14(30)	1.18 ± 0.14(32)	*	Present study
	0.67 ± 0.06 (5)	0.97 ± 0.06 (5)	**	54
	1.02 ± 0.09 (8)	1.23 ± 0.15 (8)	+	42
	0.80 ± 0.05(10)	1.29 ± 0.09(10)	*	399
Nucleus accumbens	0.69 ± 0.18(43)	1.40 ± 0.22(43)	*	Present study
	0.26 ± 0.02(20)	0.90 ± 0.03(18)	**	47
		2.20 ± 0.17 (6)	***	400
	0.61 ± 0.16(10)	1.23 ± 0.10(10)	*	399
Olfactory tubercle	0.76 ± 0.16(16)	1.41 ± 0.20(18)	*	Present study
	0.17 ± 0.02(17)	1.03 ± 0.02(20)	**	47
	0.52 ± 0.14 (5)	1.14 ± 0.15 (5)	*	41
Hypothalamus	0.20 ± 0.04 (6)	0.31 ± 0.04 (6)	*	Present study
	0.09 ± 0.01 (8)	0.24 ± 0.06 (8)	+	42
Cerebral cortex	0.12 ± 0.05 (9)	0.11 ± 0.03 (9)	*	Present study
	0.02 ± 0.02 (5)	0.06 ± 0.01 (9)	**	401
	0.12 ± 0.01 (8)	0.13 ± 0.01 (6)	+	42
Hippocampus	0.23 ± 0.03 (7)	0.24 ± 0.02 (8)	*	Present study
	0.16 ± 0.01 (4)	0.18 ± 0.01 (3)	+	42
Frontal cortex	0.18 ± 0.02(21)	0.14 ± 0.02(21)	*	Present study
	0.02 ± 0.03(13)	0.06 ± 0.01 (8)	**	401
Substantia nigra	1.50 ± 0.25(26)	1.22 ± 0.27(26)	*	Present study
		1.71 ± 0.22 (6)	***	400
Cerebellum	0.05 ± 0.01 (5)	0.05 ± 0.01 (5)	*	Present study
	0.03 ± 0.01 (5)	0.07 ± 0.01 (5)	+	42

Results are the means ± s.e.m. (s.d. in the present study) of values obtained from the number of rats shown in parentheses.

Method of estimation:
- * Gas chromatography
- ** Fluorimetry
- *** Radioisotope
- + Mass fragmentography

other workers (23). However, in order to clarify this point, more complex measurements must be carried out, which would include the total HVA and DOPAC content, free and conjugated (21,24). The relative importance of the two metabolites in the various brain regions can only be assessed if the rates of synthesis, metabolism and transport for both free and conjugated HVA and DOPAC are determined.

The results shown in Table 1.1 also indicate that DA metabolism is not uniform in brain and furthermore that it is different in different parts of a neuron. The presence of DA-containing cell bodies in substantia nigra (5) and the presence of DA nerve terminals in corpus striatum (8), olfactory tubercle (8,9) and nucleus accumbens (8,9) is well documented. The presence of DA neurons in the hypothalamic area (10), in the cerebral (48,49) and in the frontal cortex (9,49) has also been established. Therefore, the finding of relatively high concentrations of DA metabolites in these areas is not surprising. The DAergic cell bodies of the mesencephalic brain areas seem to be the origin of the DAergic innervation of these regions. The nerve terminals of the striatum are known to originate from the area A9 substantia nigra (8). The terminals in the nucleus accumbens and olfactory tubercle originate from the area A10, medial to the substantia nigra (8,9). Lesion studies indicate that the areas A9 and A10 probably give rise to the projection to the frontal cortex and the cerebral cortex (9,49). The tubero-infundibular DAergic pathway is more likely to be the source of innervation of the hypothalamic area (4,10). The possibility must be considered that HVA and DOPAC detected in areas not previously associated with DAergic innervation might derive from metabolism unrelated to DAergic neurotransmission. This is probably the case in the cerebellum, where there is apparently no DAergic innervation (8) or DA receptors (50); the DA present in this structure might function as a precursor of noradrenaline.

The presence of nerve terminals containing DA in the hippocampus(63) explains the finding of moderate amounts of HVA and DOPAC in this structure. The finding of high concentrations of HVA and DOPAC in the substantia nigra is consistent with the high tyrosine hydroxylase activity found in this structure (81). However, the functional significance of the DA metabolites in the cell body area of substantia nigra could be assessed only after pharmacological or other manipulations which could alter selectively their concentration, and by comparison of these effects with the effects at the nerve terminal areas.

1.4.2 Drug effects on DA metabolism in corpus striatum and substantia nigra

1.4.2.1 Support for a neuromodulatory function of DA in the substantia nigra

Research on central DA neurotransmission was, until very recently, almost exclusively restricted to axon nerve terminals. The histological studies of Rinvik and Grofova (51), of Hajdu et al. (32) and of Björklund and Lindvall (31) have, however, revealed the presence of DA in dendrites of the substantia nigra, a region known to contain DAergic cell bodies. These dendrites contain relatively large amounts of DA, revealed by histofluorescence, and vesicle-like structures (32). DA seems to be stored in reserpine-sensitive structures (31), which are also able to take up ^{3}H-DA (31,51). Exogenous ^{3}H-DA taken up in slices of rat substantia nigra was shown to be released by potassium (30 mM) through a calcium-dependent process (34). Furthermore, spontaneous and evoked in vivo release of ^{3}H-DA has been demonstrated from the cat substantia nigra (52). These observations, together with the presence of a DA-sensitive adenylate cyclase in the substantia nigra with characteristics similar to that in the striatum (18), where it is apparently associated with the DA-receptors (16), suggest

that DA may serve a neuromodulatory function in this structure. The suggestion has been made that in the substantia nigra DA released from dendrites or cell bodies may influence the activity of the nigrostriatal DAergic neurons by interacting with dendritic or somatic DA receptors, which have been termed autoreceptors (53,54). However, the finding that the DA-sensitive adenylate cyclase is apparently located on the terminals of striato-nigral afferents containing either GABA or substance P (56, 46), and the demonstrated in vitro release of GABA from superfused slices of rat substantia nigra both by DA and by amphetamine (57) suggest that the proposed local dendritic release of DA may modify the excitability of DAergic neurons by stimulating the local release of GABA. On the basis of these results, it has been concluded that the activity of the DAergic neurons is inversely correlated to the extent of the dendritic release of the transmitter.

1.4.2.2 Possible mechanisms of auto-inhibition of DAergic cells in the substantia nigra

Westerink and Korf (54) have demonstrated that various treatments with drugs thought to affect primarily DA neurotransmission in brain produce parallel changes in HVA and DOPAC levels in both substantia nigra and corpus striatum; the apomorphine- and haloperidol-induced changes in the substantia nigra were thought to result from DA autoreceptor stimulation or blockade, respectively.

The present study confirmed that parallel changes in the levels of the two acidic metabolites of DA may occur in both striatum and substantia nigra. There was a significant decrease in the concentrations of the two metabolites in both structures at 20 min after i.p. injection of apomorphine (1 or 2 mg/kg), indicating the presence of

DA receptors in striatum and substantia nigra and the existence of similar mechanisms leading to a decrease in DA metabolism, induced as a consequence of DA receptor stimulation. However, when the DA receptor stimulant was administered to the rats 10 min before sacrifice, significant increases in HVA and DOPAC were found in the substantia nigra, in contrast to the significant decrease found in the striatum. Thus the belief that DA receptor stimulation produces a marked inhibition of activity in the DAergic neuronal terminals and a depression of firing of the cell bodies of the DAergic neurons in the substantia nigra (53) cannot be always supported by the findings of this study. The initial increase in DA metabolite concentrations in the substantia nigra after apomorphine may indicate an initial stimulation of firing rate in this structure, resulting in increased dendritic release of DA, which in turn may act directly or through release of an inhibitory transmitter (e.g. GABA), causing finally an inhibition of neuronal firing. This sequence may explain the reduction of HVA and DOPAC that follows the rapid initial increase in the substantia nigra.

An apparent discrepancy arises, however, between the previously described inhibitory effects of DA receptor agonists on the firing rate of the cells in the substantia nigra and the hypothesised initial stimulation of the firing rate by apomorphine. This discrepancy could be resolved if any of several other possibilities were assessed. First, the proposed existence of two populations of DA receptors in the striatum and the nucleus accumbens of the rat brain, termed excitation-mediating DA receptors (DAe) and inhibition-mediating DA receptors (DAi) (55) might be considered in relation to the DA autoreceptors believed to exist in the substantia nigra. These autoreceptors may be subdivided into two groups, DAe and DAi, of which DAe may be selectively

activated during the initial period and DAi during the subsequent period. This explanation would be simpler than those involving dendritically released DA or DA-induced release of GABA as mediators of the autoregulation of the DA cells, but there is no direct or indirect evidence in favour of this hypothesis. Second, stimulation of the release of an excitatory transmitter such as substance P or acetylcholine from their neurons, mediated through stimulation of DA receptors located on the respective neurons, might explain the initial stage of the biphasic effects of apomorphine. The existence of a DA-sensitive adenyl cyclase on nerve terminals containing substance P has been demonstrated in the substantia nigra (46). An initial stimulation of the release of substance P or acetylcholine, known to be excitatory on cell activity in substantia nigra (64) may be responsible for the demonstrated transient increase in HVA and DOPAC following apomorphine administration. Subsequent stimulation of the DA autoreceptors may surmount the effect of the excitatory transmitter. Third, two forms of adenylate cyclase may exist: one located on DA cell bodies or dendrites, and the other located on GABA- or substance P-containing neurons. Preferential stimulation of the DA receptors operating through either of these adenylate cyclases may be followed by stimulation of the other receptors resulting by an unknown mechanism to inhibition of the cell firing. Fourth, the existence of two types of DA autoreceptors, one on the DA cell bodies and the other on the DAergic dendrites. Initial stimulation of the 'dendritic' autoreceptors may be responsible for the release of DA and the observed initial increase in HVA and DOPAC, whereas subsequent stimulation of the 'somatic' autoreceptors may be responsible for the prolonged cell inhibition and the parallel fall in the concentrations of the metabolites of DA in the substantia nigra.

1.4.2.3 Different responses of the striatum

These and other possibilities, available for consideration, may also be examined in order to interpret the biphasic effects of haloperidol on the concentrations of HVA and DOPAC in the substantia nigra, which are in general the reverse of those following apomorphine (Fig. 1.2 and 1.3). On the other hand, the different responses of the substantia nigra and the corpus striatum to apomorphine and haloperidol indicate that local regulation of DA utilisation may occur in both structures, rather than the regulatory system operating through a neuronal striatonigral feedback loop proposed by Carlsson and Lindquist (44). In this case, different mechanisms may operate in the two structures. However, the possibility exists that the cell body area and the terminal area have the same regulatory mechanism, the initial increase in DA metabolism, observed 10 min after apomorphine, being a latency phenomenon with unknown physiological significance. Support for this hypothesis comes from electrophysiological experiments (M. Garcia-Munoz, personal communication) which showed that intravenous administration of apomorphine or amphetamine causes an initial increase in firing rate of DA cells in the substantia nigra, rapidly followed by a prolonged inhibition. This biphasic effect on firing rate might be the reason for the biphasic effect on DA metabolite levels observed in the substantia nigra after apomorphine.

The question why changes in the firing rate of the DA cells in the substantia nigra are not followed by appropriate changes in impulse flow to the nerve terminals (implied by the differential effects of apomorphine and haloperidol on HVA and DOPAC in the substantia nigra and the corpus striatum), may be answered (if the nigral regulation of striatal DA metabolism is accepted) by the fact that a nerve impulse

is generated only when the threshold of stimulus is exceeded. Therefore, subtle changes in cell firing rate may not result in changes in the impulse flow to the nerve terminals. This idea could facilitate the interpretation of results of our subsequent experiments (described in other Sections) and also of reports in the literature providing evidence that changes in DA metabolism in the substantia nigra are followed by no changes or even by changes in the opposite direction in the corpus striatum. As mentioned previously, iontophoretic application of DA in the substantia nigra causes a decrease in the firing rate of the DA cells, thought to result from a direct action on DAergic cell bodies (67) and to be mediated by presynaptic autoreceptors localised on the DA cell bodies (53). Presynaptic DA autoreceptors have also been postulated to exist on the striatal DAergic nerve terminals (63). Further studies are needed in order to clarify whether DA autoreceptors exist on DA cells or dendrites in the substantia nigra, whether in addition to DA autoreceptors, GABA or other receptors exist on DA cell bodies or dendrites mediating in part or totally the inhibitory effect of DA on cell firing, or whether local neuromodulation takes place and to what extent in the activity of the nigral neurons.

1.4.2.4 Effects of amphetamine on DA metabolism in the substantia nigra and the striatum

Intraperitoneal administration of amphetamine produced a depression of neuronal activity in the substantia nigra (53), possibly through a mechanism intrinsic to this structure. Consistent with this is the finding of a decrease in the levels of both HVA and DOPAC in the substantia nigra 1 hour after the administration of amphetamine (Table 1.2). In the striatum on the other hand, the same dose produced no effect on HVA, but a significant decrease in DOPAC levels, confirming the suggested

effects of this agent on DA release and reuptake (43) and the proposed intraneuronal formation of DOPAC and extraneuronal formation of HVA (38,39,40).

In behaviourally effective doses the primary action of amphetamine in brain appears to be that of increasing the release and blocking the reuptake of catecholamines, thereby indirectly stimulating postsynaptic DA receptors (43). In 1967 Corrodi and co-workers suggested that this action of amphetamine on postsynaptic neurons causes a compensatory decrease in the firing rate of DAergic (presynaptic) neurons via a neuronal feedback pathway.

Intravenous injection of amphetamine in low doses inhibited DA cell activity in the substantia nigra (53,59). Direct application of amphetamine onto DA cell bodies and proximal dendrites as well as on distal dendrites produced only a decrease of approximately 25% in DA cell activity (59). The effect of amphetamine on cell firing in the substantia nigra was reduced by lesions of the descending striatonigral pathway (61). The above evidence seems to support the hypothesis first proposed by Corrodi et al. in 1967 that the depressant effect of amphetamine (in pharmacologically relevant doses) on DAergic cells is mediated primarily through its effect on a striatonigral feedback pathway (60). The finding that the amphetamine-induced inhibition of DAergic cells is reversed by an intravenous injection of picrotoxin (59) suggested that such a pathway may contain a neuron which uses GABA as its neurotransmitter.

1.4.2.5 <u>GABA as possible mediator of auto-inhibition in the substantia nigra</u>

However, the idea of a GABAergic neuronal feedback loop regulating the activity of the nigrostriatal DAergic pathway has been

challenged (45). Further reports seem to offer alternative interpretations of the above data, which do not depend on the existence of a neuronal striatonigral feedback pathway. The finding that DA at concentrations $5 \times 10^{-6} - 5 \times 10^{-4}$ M and amphetamine can selectively release GABA from nigral slices in vitro by a neuroleptic-inhibited process (57), combined to the localisation of DA-sensitive adenylate cyclase in non-DAergic neurons of the substantia nigra (18,62), probably GABA-containing in nature (56), may suggest that the inhibitory effect of GABA on DA cells or dendrites follows the release of GABA from its neurons by a DA-sensitive process. Our finding that amphetamine increases the turnover of GABA (Section 3) while decreasing (probably as a consequence) the DA turnover in the substantia nigra provides further support for this hypothesis. The different effects of various treatments with DA receptor agonists and antagonists on the GABA turnover in the striatum and the substantia nigra seem to support local links interrelating the DA and GABA systems, in both structures. The functional integrity of the nigrostriatal or the striatonigral pathways does not always appear to be critically involved in the action of DA receptor blockers or stimulants on the metabolism of DA in the striatum or the substantia nigra (45).

The presence of presynaptic DA receptors at the nerve terminals which appear to regulate the rate of DA synthesis and turnover by a local negative feedback mechanism (44) is supported by various pharmacological or other treatments. Stimulation of these receptors by DA agonists, such as apomorphine, caused a receptor-mediated reduction in DA synthesis and blockade by DA antagonists, such as haloperidol, resulted in an increase in DA synthesis (25). Parenteral or microiontophoretic administration of DA agonists and antagonists produced

physiological responses of DAergic neurons in the substantia nigra (pars compacta) which paralleled the biochemical effects at the terminal area (67).

DA receptor agonists seem, according to the proposed model, to act in the substantia nigra as GABA-like agents, thereby reducing the DAergic cell firing rate. The opposite appears to happen with DA receptor antagonists. The involvement of DA-DA interactions, as well as the involvement of substance P or acetycholine at certain stages of the nigral DA neuromodulation may also be proposed to occur in parallel with the GABA-mediated control of DA neuronal activity. A combination of these mechanisms might explain the finding of an inverse correlation between the activity of the DAergic nigrostriatal pathway (as shown by changes in the turnover of DA in the striatum) and the extent of the dendritic release of DA in the substantia nigra (as shown by changes in the turnover of DA in the substantia nigra). The demonstrated similarities between the two structures can also be interpreted on the basis of the proposed local regulation of DAergic neuronal activity. The various possibilities are depicted in a generalised form in Fig. 1.5.

1.4.2.6 Haloperidol treatment: support for local regulation of DA neuronal activity

The time-effect curves as well as the dose-effect curves of the haloperidol treatment, demonstrate striking differences in the response of the striatum and the substantia nigra with respect to changes in HVA and DOPAC concentrations. Haloperidol at a dose of 1 mg/kg produced within 30 min an initial significant decrease in the levels of the metabolites in the substantia nigra, which was followed by a sharp increase within the next 30 min. Within the subsequent

2 hours the metabolite concentrations had fallen to control values. The concentration of DOPAC was even significantly lowered 3 and 5 hours after injection, whereas the HVA concentration was not different from control at this time. On the other hand, in the striatum, there was a rapid increase in the concentrations of HVA and DOPAC which persisted for 2 hours and then declined to normal levels by 5 hours after the injection. Thus, the finding that haloperidol increased the activity of DAergic cells in the substantia nigra (e.g. 59) cannot alone explain the difference in the HVA and DOPAC concentrations in the two areas. Changes in impulse flow should result in parallel effects on DA metabolism in the substantia nigra and the striatum, if there is a single regulatory mechanism for DA metabolism. This similarity appeared only at certain times, and as the Fig. 1.2 shows, only at certain doses. In contrast to the biochemical events in the striatum, where increasing increments in the concentrations of HVA and DOPAC are obtained at 1 hour after increasing doses of haloperidol within the range 0.4-10 mg/kg, in the substantia nigra the significant increase of the acid metabolites observed with doses of 0.4-2 mg/kg was less and insignificant at higher dose levels of 5-10 mg/kg.

If the blockade of postsynaptic DA receptors in the striatum was followed by the inactivation of the "feedback" striatonigral pathway, leading to an increase in the activity of the DA cells in the substantia nigra (44, 60), the striatal DA turnover would consequently increase, but a parallel increase would be observed in the nigral DA turnover. This sequence was not found in the present study. Therefore, an interpretation based on the existence of local regulatory mechanisms in both structures seems to be supported by the differential effects of haloperidol on HVA and DOPAC in the two areas. Blockade of

postsynaptic DA receptors in the striatum may lead to an activation of a local feedback mechanism resulting in increased DA synthesis and turnover, as it was supported by previous studies (63). Blockade of DA receptors in the substantia nigra, probably located on terminals of striatonigral GABA-containing neurons (56) forming synapses with the DA-containing dendrites, may prevent the release of GABA which normally acts to inhibit the DA cells or the dendrites. Therefore, the DA cells or the dendrites are released from the inhibition during DA receptor blockade and respond to this treatment with an increase in DA turnover.

This mechanism does not explain either the initial fall in DA turnover or release implied by the significant fall in HVA and DOPAC after haloperidol or the reduction of the response with higher doses of haloperidol. The latter may result from the activation of an alternative mechanism, such as the proposed autoreceptor-mediated regulatory system, (53) which may obscure the increase in DA turnover. Although there is no obvious explanation for the decrease in HVA and DOPAC in the substantia nigra observed 30 min after haloperidol, a blockade of DA autoreceptors leading to a subtle decrease in the cell firing rate not evident in the striatum (contrary to an initial activation by apomorphine leading to an increase in the cell firing rate), seems to be a possible mechanism functioning in parallel with the proposed GABA-mediated local regulatory system.

The various possibilities considered above in relation to the effects of apomorphine on HVA and DOPAC in the substantia nigra may also be proposed. Furthermore, the finding of Westerink and Korf (47), that haloperidol induced a rapid increase in HVA and DOPAC in the substantia nigra 10 min after the injection, which within another

5 min disappeared, may also be relevant to the decrease found in the present study at 30 min. Thus, the increased release or turnover of DA implied by the temporary rise in HVA and DOPAC at 10 min may result in an inhibition of the cell firing rate and a subsequent reduction in the release or turnover of DA reflected in the reduction of the concentrations of the metabolites found in the present study at 30 min after administration of haloperidol. Finally, the possibility that DA is metabolised under certain circumstances via the 3-methoxytyramine pathway (44) and therefore HVA and DOPAC do not always reflect the activity of DAergic neurons cannot be excluded.

1.4.2.7 Alternative interpretations

Several other, rather remote, possibilities could be considered in attempting to explain the different biochemical responses of the substantia nigra and the corpus striatum after various treatments. First, different distribution or access to the receptor sites may be responsible for some of the observed different effects of drugs at certain doses and time-intervals. The fact, however, that some of the effects of DA receptor agonists are in the opposite direction that the effects of DA receptor antagonists eliminates this possibility and suggests that the main effects are mediated through a direct action on DA receptors. A study of the distribution of haloperidol, apomorphine and amphetamine in the striatum and in the substantia nigra could clarify this point. Second, a difference between the characteristics of the DA receptors found in the two structures may account for the different responses. A different affinity of the drugs for these receptors might imply that DA receptors in the striatum are different from DA receptors in the substantia nigra. Although the adenylate cyclase found in the substantia nigra appears to have characteristics similar to that in the striatum,

which is thought to be associated with DA receptors (16), it is possible that the nigral DA-sensitive adenylate cyclase is not associated with DA receptors, or that there are more than one adenylate cyclases in the substantia nigra. Third, effects of these drugs on blood flow (65) or glucose utilisation (66), which may affect the distribution or the clearance of the drugs or the metabolic rate of the neurons, may contribute to the effects of the treatments on DA metabolism. Fourth, the involvement of other neurotransmitter systems, such as the GABAergic, the cholinergic and the serotonergic system, may critically determine the response of the DAergic system in the two regions. These possible mechanisms are examined in subsequent Sections of the present Thesis, where the proposed model of regulation of DAergic neuronal activity is tested by the use of other pharmacological or lesioning procedures.

1.4.2.8 Conclusions

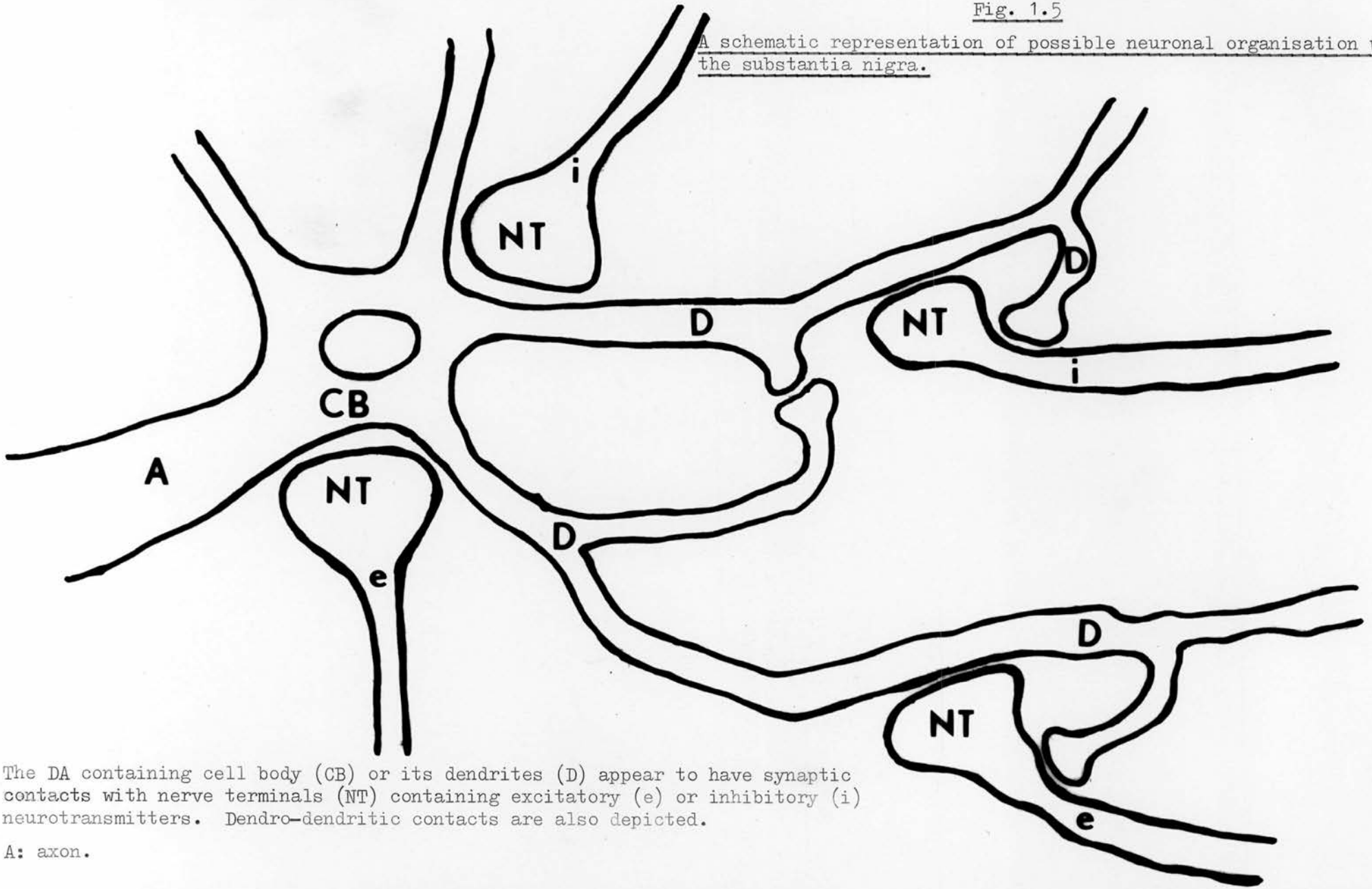
Taken together the results reported in this section indicate that changes of DA turnover in the rat substantia nigra are not, in general, followed by changes in the same direction in the corpus striatum; at certain time and dosage, the opposite effects of DA receptor agonists and antagonists are seen in the two areas, i.e. an increase in DA turnover in the corpus striatum is associated with a decrease in the substantia and vice-versa.

The findings suggest that local independent mechanisms for the regulation of DA metabolism may exist in the substantia nigra and the corpus striatum. The DA 'autoreceptor' theory does not explain sufficiently these results, neither does the striatonigral 'neuronal feedback loop' hypothesis. Modulation of DAergic cell activity and DA release from the dendrites of the substantia nigra, mediated by inhibitory (e.g. GABA, 5-HT) or excitatory (e.g. substance P, Ach) transmitters,

may be postulated to replace or accomplish the proposed DA autoregulation. An embryonic model integrating the most probably mechanisms for the modulation of DA cell activity in the substantia nigra is illustrated in Fig. 1.5. It appears that the dendro-dendritic contacts and the postulated DA-induced release of certain excitatory or inhibitory transmitters may result in the appropriate effect on DA cell activity and finally DA release from the dendrites.

Fig. 1.5

A schematic representation of possible neuronal organisation within the substantia nigra.



The DA containing cell body (CB) or its dendrites (D) appear to have synaptic contacts with nerve terminals (NT) containing excitatory (e) or inhibitory (i) neurotransmitters. Dendro-dendritic contacts are also depicted.

A: axon.

SECTION 2

SOME BIOCHEMICAL EFFECTS OF LESIONS IN THE SUBSTANTIA NIGRA OF THE RAT BRAIN WITH 6-HYDROXYDOPAMINE

2.1 INTRODUCTION

2.1.1 Substantia nigra: anatomy, biochemistry

The dopamine (DA)-containing cell bodies of the substantia nigra are found densely aggregated throughout the pars compacta and scattered in the pars reticulata (31,51). Their axons are known to leave the nigral cells and form the medially directed DA fibre-outflow of the substantia nigra, ascending partly within the medial forebrain bundle (7,8,9) and terminating within the corpus striatum, where they form a dense terminal plexus (8,9,31). In Golgi and histofluorescence studies (31,51), the nigral cells have also been shown to project abundantly with several long processes which ramify within the pars reticulata. These processes fulfil the ultrastructural criteria for dendrites (32,51).

It has been demonstrated that both the axonal and dendritic terminals in the substantia nigra possess storage and uptake mechanisms which are reserpine-sensitive (31). It has also been shown that DA may be released from the cell-bodies and dendrites of the substantia nigra as well as from the corpus striatum after electrical stimulation (33) or potassium depolarisation (34). It seems that DA released from the dendrites can be partly inactivated by a potent reuptake process as is observed in the nerve terminals. The DAergic dendrites contain tyrosine hydroxylase, the rate limiting step in the synthesis of DA, as indicated by a recent immunchistochemical study (77). Other results suggest that the local synthesis rate of DA in the dendrites is important and can efficiently supply the release process

(52). When compared to the striatal axon terminals the dendrites show a relatively much greater uptake and retention of ^{3}H-DA (34), probably because of a lower endogenous DA content and a higher storage capacity for exogenous DA in the vesicles of dendrites.

2.1.2 Physiological role of dopamine released in the substantia nigra

It has been postulated that the demonstrated release of DA from dendrites in the substantia nigra has important physiological functions. Firstly, DA may inhibit the neurons from which it is released (self- or auto-inhibition). In microiontophoretic electrophysiological studies it has been found that DA-containing cells of the substantia nigra are inhibited when the amine is applied to the pars reticulata (67), and it has also been postulated that DA acts on DA autoreceptors on the cells, thus controlling the range of their firing frequencies (53). Secondly, DA released in the substantia nigra may inhibit other neurons located near the dendrites releasing DA (lateral inhibition). Dendro-dendritic contacts in the substantia nigra have been revealed by electron microscopy (32), and it could be postulated that an action of DA on receptors located either on the DA dendrites or presynaptically on the axon terminals innervating them may take place. A third regulatory function of the dendritic release of DA could be the control of plastic regenerative sprouting of neuronal processes and the formation of new synapses (33).

The local application of DA or DA receptor agonists to the substantia nigra produces an inhibition in the firing of neurons in the pars compacta, which is blocked by DA antagonists (53,67). This finding does not discriminate between the possible functions of DA released from the dendrites.

2.1.3 Striatal and nigral interneurons

Neurons containing γ-aminobutyric acid (GABA), substance P, acetylcholine (Ach) and 5-hydroxytryptamine (5-HT) have been found in the substantia nigra, but relatively little is known about the synaptic interactions of these neurons (see review by Dray and Straughan, ref. 64).

The very high concentration of GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) in the substantia nigra is well established (81,69). The main projection to the substantia nigra appears to arise unilaterally from the corpus striatum (84,68), is mainly inhibitory (81,82) and utilises GABA as its neurotransmitter (68). The striatonigral projection also contains an excitatory pathway which may utilise substance P as its neurotransmitter (58). Moreover, GABA-containing striatal neurons which travel through the globus pallidus on their way to the substantia nigra have been described (84). Evidence has been presented that the striatonigral pathway terminates mainly within the pars reticulata (85), where GAD activity is higher than in the pars compacta (86).

Fibres arising from cell bodies in the raphe nuclei terminate within the substantia nigra, and probably utilise 5-HT as their neurotransmitter (73,87). Evidence has been presented for synaptic contacts between the 5-HT-containing nerve terminals and the DAergic neurons in the substantia nigra (73).

It is well established that the nigrostriatal DAergic neurons exert a tonic inhibition of certain striatal cells through the release of DA as a neurotransmitter. Morphological evidence suggests that synaptic boutons belonging to the DAergic terminals exist in the striatum (88). In addition, stimulation of the nigrostriatal pathway causes

release of DA from the striatum (89). Electrical stimulation of the substantia nigra induces mainly inhibition of the activity of certain striatal cells, presumably by a direct monosynaptic process (90,91). Moreover, local iontophoretic application of DA to the striatal cells, which are inhibited after nigral stimulation, also causes a depression of firing rate (90,92). The above observations lead to the view that DAergic postsynaptic receptors are present on certain striatal neurons and mediate the inhibitory effect of DA, which is released by the DAergic terminals. The cells which form synapses with the DAergic terminals appear to be cholinergic (93). Anatomical evidence has also been presented, showing that the DAergic nerve endings make synaptic contacts with cholinergic dendritic structures which contain the Ach-synthesising enzyme choline acetyltransferase (CAT) (95,96).

Lesioning studies suggest that the cholinergic system in the striatum may also be primarily organised within this structure (97) and that the described afferent from the ventral tegmentum (98) or the efferents to the globus pallidus and the substantia nigra (99) may not be responsible for the cholinergic activity in the striatum. Cholinergic neurons seem to have short axons within the striatum, with many terminals which could contain most of the Ach and CAT activity. The striatum contains the highest CAT activity in the brain (100,101), mostly concentrated in nerve endings (101).

GAD, the rate-limiting enzyme in the synthesis of GABA, is one of the best available markers for GABAergic neurons. The activity of GAD correlates well with the distribution of GABA (75,76) and during ontogenesis parallel increases in GAD activity and GABA concentration can be observed (102). GAD is found mainly in synaptosomes (76), which suggests that GABA formation may occur in nerve endings in vivo.

Furthermore, GAD, unlike GABA, is much less subject to post-mortem changes (86). In many inhibitory nerves, both GAD and GABA are present and are distributed throughout the neuron, although the GAD is somewhat more highly concentrated in the presynaptic endings than elsewhere. The GABA-transaminase is contained in the mitochondria of all neuronal regions, but it seems to be richer in the mitochondria of those neuronal sites onto which GABA might be liberated (for review see ref. 104). Such regions would be expected to exist in pericarya and dendrites that receive inhibitory inputs and possibly in the glial and endothelial cells which are in the vicinity of inhibitory synapses (104).

Recent pharmacological findings suggest that DAergic neurons can control GABA neurons in the striatum. Thus, the DA receptor agonist apomorphine can increase the GABA turnover in the striatum and this effect can be counteracted by the DA receptor blocking agent pimozide (105). DA has been demonstrated to have mainly an inhibitory action on the cells in the striatum (90,92), and therefore the DA terminals may not have a direct contact with the GABAergic cell bodies giving rise to the descending striatonigral pathway, since an increase of GABA turnover has been demonstrated following DA receptor stimulation. Cholinergic interneurons may be involved in the control of both the descending GABA pathways and the GABA interneurons within the striatum itself (106).

2.1.4 Lesions of the nigrostriatal pathway

It is possible to study the interactions between the various neuronal systems and to elucidate the neuronal circuitry of a region such as the striatum, by selectively manipulating one system and

examining the effect on the others. Lesioning of the substantia nigra, where the cell-bodies of the DAergic neurons terminating in the striatum are located, has been frequently employed in the study of the functional importance of the nigrostriatal DAergic pathway in the control of behaviour. Unilateral intracerebral injection of the neurotoxin 6-hydroxydopamine (6-OH-DA) directly into the DAergic cell-body region of the substantia nigra of a rat induces a selective degeneration of the entire nigrostriatal DAergic neuronal system and disappearance of DA from the striatum (107). The lesion causes deviation in the animal's movements and posture towards the lesioned side (107,108); this has been attributed to the imbalance in DA neurotransmission in the two sides of the brain (8). DA releasing drugs, e.g. amphetamine, induce a dose-dependent rotation of the rat towards the side with the lesion, whereas DA receptor stimulating agents, such as apomorphine, induce marked dose-dependent rotation towards the non-lesioned side (110). The fact that the animal moves away from the side where the DA receptors are most stimulated, demonstrated by local injections of DA into the striatum (111), indicates that the rotation after the administration of apomorphine is due to an increased sensitivity of the striatal postsynaptic DA receptors ('supersensitivity') on the denervated side, whereas the amphetamine-induced rotation is due to the release of DA from the intact side of the striatum only. Low doses of neuroleptic agents, i.e. DA receptor blockers, inhibit the turning behaviour induced by DAergic receptor agonists (110). Cholinergic receptor agonists block the turning induced by DAergic agonists, whereas anticholinergic agents induce turning towards the side with the lesion (112). These findings suggest that the synaptic contacts of the nigrostriatal pathway

at the striatal or nigral level may play a part in the circling behaviour of rats with specific lesions of the DAergic neurons.

2.1.5 'Supersensitivity' of DA receptors after denervation

If receptors in the peripheral nervous system are deprived of their natural chemical transmitter for some time, they develop supersensitivity to this substance (113). The same reason has been advanced to explain the 'supersensitivity' phenomena after lesioning of the nigrostriatal pathway in the central nervous system, e.g. the supersensitivity of striatal postsynaptic DA receptors of the denervated side to systemically administered DA receptor agonists or iontophoretically administered DA (114).

However, the increased responsiveness to DA receptor stimulants after nigrostriatal lesions might result from changes distal to the DA receptor or in other systems. Alternatively, it could reflect a true alteration of the DA receptor itself. Activity of a striatal DA-sensitive adenylate cyclase, which appears to be associated with the DA receptor, has been reported to be unaffected by nigrostriatal lesions (115) or to be enhanced (116). It should be mentioned that the adenylate cyclase activity in the substantia nigra is not affected after 6-OH-DA lesions of this structure but it disappears after lesions of the striato-pallido-nigral GABAergic or substance P-containing connections (56, 18). Enhanced DA receptor binding of (^{3}H)-haloperidol has been found in the denervated striatum (119). This increased number of DA receptors could account for the behavioural supersensitivity to DA agonists which develops after lesions of the substantia nigra. Electrophysiological experiments produced evidence that the striatal cells on the lesioned side

show higher firing rates than those on the control side (120). In addition, the cells of the lesioned side respond to DA receptor agonists (apomorphine and L-dopa) with a much greater reduction in their firing rate than the cells on the intact side (121).

The biochemical, pharmacological and behavioural events observed after nigrostriatal lesions should be interpreted with caution, for two main reasons:

(a) A close anatomical relation seems to exist between the two nigrostriatal systems (in the two sides of the brain), probably through a nigro-thalamo-cortical pathway, which in turn may project to the contralateral striatum and indirectly to the nigra through the striatonigral pathways. Thus, a lesion made in the left substantia nigra of the cat blocks the release of (^{3}H)-dopamine in the ipsilateral striatum, but an increase in the release of (^{3}H)-dopamine in the contralateral side is observed. The opposite effect is produced by the application of DA in the left substantia nigra, (122).

(b) Remaining DAergic neurons after partial destruction of the nigrostriatal pathway are hyperactive, with increased DA synthesis and utilisation (123). This is probably caused by a compensatory mechanism induced by the DA deficiency at some postsynaptic receptor sites.

2.1.6 'Supersensitivity' in Parkinson's disease

In addition to its usefulness in the study of the nigrostriatal neuronal connections, in the screening of potential DA agonists and antagonists and in the study of the involvement of DA in the motor behaviour, the rat rotational model has been proposed as a model of Parkinson's disease (8). It has been suggested that the progressive death of the substantia nigra cells and degeneration of the nigrostriatal

DAergic pathway that occurs in Parkinson's disease (117) may cause denervation supersensitivity of striatal DA receptors. The effectiveness of L-dopa in the treatment of this disease can thus be explained by the idea that the DA synthesised from L-dopa acts predominantly on the supersensitive receptors of the denervated striatum (110).

2.1.7 Effect of DA receptor agonists and antagonists on other neuronal systems

All available evidence seems to suggest that, in the striatum, DA exerts an inhibitory action on cholinergic mechanisms, whereas acetylcholine (Ach) has, possibly by way of a feedback, an enhancing action on striatal DAergic mechanisms. Thus, drugs which are supposed to selectively block DA receptors in the striatum (such as most of the neuroleptic agents) as well as cholinergic agonists, increase the synthesis and release of DA in the striatum (54, 118); the former drugs also increase the Ach release or decrease its levels (124,125). Conversely, compounds with DAergic agonist action (L-dopa, amphetamine, apomorphine) decrease the Ach release in this region (124,125). In addition, anticholinergic agents (in high doses) seem to cause a decrease in striatal DA turnover (126) and cholinergic agonists, such as oxotremorine, to cause an increase (54).

As suggested by Kebabian and Greengard (127) for the peripheral autonomic ganglion, stimulation of DA receptors produces post-synaptic inhibition resulting in hyperpolarization (30) and decreased release of Ach. This latter effect could lead to accumulation of transmitter in striatal interneuronal cholinergic nerve terminals, and consequently the levels of Ach would be higher. Similarly, blockade of DA receptors produces disinhibition of the cholinergic interneurons, which leads to their overactivity and a marked release of Ach, and consequently decreased

levels of this transmitter.

Pharmacological evidence suggests the possibility of a DA-GABA interrelation at the level of the basal ganglia (129). It has been shown that drugs increasing GABA concentrations in the brain reduce DA turnover in the striatum (130,131). Conversely, prolonged administration of L-dopa increases the activity of the GABA-synthesizing enzyme GAD in the striatum (132), indicating an enhancing effect on the GABAergic activity.

From these and other studies, the conclusion may be drawn that the interactions between the neuronal systems play an important role in the normal function of the basal ganglia and that disturbance of one of these systems will frequently be followed by changes in the others, which can be predicted.

2.1.8 GABA: post-mortem changes

GABA appears to increase rapidly in brain tissue after death. Since Elliot and Florey, in 1956 (133) observed that the GABA content of brain tissue increased after excision, there have been several conflicting studies dealing with the magnitude and time-constant of this increase. Lovel and Elliot (134) observed increases of about 30 per cent, 2 min after decapitation, followed by a steady but slower increase. Minard and Mushahwar (135) reported that GABA levels reached a plateau within 1-2 min, without further increase. Shank and Aprison (136) reported changes in several brain areas as early as 30 sec and lasting for at least 10 min post-mortem. Alderman and Shellenberger (137) found that a post-mortem increase in GABA levels begins at 60 sec, reaches a maximum rate of increase between 60 and 120 sec and continues until the 4th min, and then shows a plateau. The post-mortem increase found by

the last two investigators was about 30 per cent of basal levels.

Exposure to a microwave field has been proposed recently as a means of killing animals in order to inactivate enzymatic processes and prevent post-mortem changes (138). GAD appears to be totally inactivated after a 2 sec exposure of the brain to microwave irradiation, while the GABA content and distribution in various brain nuclei is not affected (86). Post-mortem increments during the first 3 min following decapitation range up to 5 times the levels measured following microwave exposure. This study (86) reported no correlation between GAD and endogenous GABA levels, but a significant correlation was found when the rats were decapitated and the tissues dissected within 3 min.

Other investigators (139) also using microwave radiation, found only 18 per cent post-mortem rise of GABA in brain tissues when the rats were decapitated and the tissues excised at room temperature within about 2 min after death. They found, in addition, much lower levels of GABA than those previously reported for brain regions, but a close correlation of the distribution to the regional distribution reported in the literature (assessed without the use of microwave fixation).

The controversy over the use of microwave irradiation as a means of killing animals and the lack of sufficient evidence for the functional importance (as well as the real extent) of the GABA formed post-mortem make the interpretation of results involving this aminoacid difficult, and its value questionable, unless other measures of the activity of GABAergic neurons are included.

2.1.9 Purpose of the present study

The concept of a functional neuronal interaction that involves DA, Ach and GABA in the substantia nigra and the corpus striatum initiated

our interest in an investigation into the effects of interruption of the nigrostriatal DAergic neuronal pathway upon the activity of neurons utilising Ach and GABA within the two structures. Also, it was of interest to study and compare the effect of drugs which are known to act primarily on DA receptors, upon DA metabolite levels (as an index of DA neuronal activity) after destruction of the cell bodies of DAergic neurons, in order to obtain some information about the site of their action. The administration of the DA receptor agonist apomorphine, in particular, known to elicit the 'supersensitivity' phenomenon in rats with lesions of the substantia nigra, was used as a tool for the biochemical characterisation of this phenomenon with regard to the DAergic system or the cholinergic or GABAergic systems supposedly linked to it functionally.

The elucidation, in particular, of the mechanism by which the GABAergic and the cholinergic system may be affecting the function of the nigrostriatal pathway, was tentatively considered and several proposed alternatives assessed in the light of the findings of this study. Although as has been consistently found by several investigators, unilateral destruction of DAergic cell bodies has a selective effect on the DAergic system in the terminal area of the striatum, the cholinergic and GABAergic systems may be in some way also affected. This secondary effect may be seen on the enzymatic activities of choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) as markers of the activity of the respective neurons or on the GABA concentrations in the striatum and the substantia nigra. In addition, further stimulation of DA receptors by apomorphine or inhibition by haloperidol might yield more information about the site and the nature of these interactions,

as well as the site of action of these agents. This would provide important clues to the overall actions of drugs thought to affect DA neurotransmission, and could guide the search for new, more specific drugs or for drugs without certain side effects.

2.2 MATERIALS AND METHODS

2.2.1 Intracerebral injection of 6-hydroxydopamine into the rat substantia nigra

Albino Wistar male rats weighing 180-210 g were used. A rat was placed in a plastic box and anaesthesia was induced by circulating a mixture of fluothane-oxygen through the box. When anaesthetised, the animal was transferred to the head-holder of a David Kopf No. 1530 stereotaxic instrument, where anaesthesia was maintained by circulating the same gaseous mixture through a vinyl mantle over the animal's nose and mouth. The concentration of anaesthetic (about 2 per cent) was adjusted to give a moderately deep narcosis at this stage. Blunt ear bars were used to fix the head position of the animal. A sagittal cut was made in the skin over the skull of the rat and the periosteum scraped off to expose the skull where the anterior suture junction, i.e. the 'bregma', is located. With the aid of a stereoscopic operation microscope (Carl-Zeiss) the injection needle was adjusted to make contact with the bregma, the co-ordinates of this point with reference to the stereotaxic instrument scales were recorded and the needle was lifted up again. The needle was then moved a predetermined distance (according to the co-ordinates chosen from Ungerstedt (8) and König and Klippel (140)) in the antero-posterior direction and in the lateral direction. A 2mm wide hole was drilled in the skull with a dental drill and the tip of the needle was lowered to a predetermined depth from the dural membrane. After the completion of the injection, the hole in the skull was sealed with bone wax following

the withdrawal of the needle and an antibiotic powder (puromycin and neomycin mixture) applied locally before suturing the scalp wound with a thread. After operation, the animals were kept warm under an infra-red lamp.

The intracerebral injections of 6-hydroxydopamine (6-OH-DA) were made routinely into the left side of the rat brain. 6-Hydroxydopamine hydrochloride (Labkemi Ab, Göteberg) was dissolved in 0.9% (w/v) sodium chloride containing ascorbic acid (1mg/ml) to prevent auto-oxidation. 8 µg of 6-OH-DA (calculated as the free base) was delivered in a volume of 4 µl. The injections were made through a 30 gauge needle which was connected to an 'Alga' micrometer all-glass syringe (Wellcome Reagents) by PE 20 polythene tubing (Portex). The 6-OH-DA solution was injected at a speed of 1 µl/min by a motor drive. 'Sham-operated' animals received an intracerebral injection of the vehicle but without the 6-OH-DA.

The co-ordinates used for animals of about 200 g were: antero-posterior (A-P)-4.2; lateral (L)-1.2; vertical (V)-7.6.

An ipsilateral spontaneous turning, as described by Ungerstedt (8) was observed on recovery from anaesthesia after the operation.

Ungerstedt (107) demonstrated that rats with a lesion of the nigro-striatal DAergic pathway or of the substantia nigra by the injection of 6-OH-DA showed anterograde and retrograde degeneration of the terminals in the caudate-putamen and of cell bodies in the substantia nigra. Animals with this type of degeneration in the nigro-striatal DAergic system showed contralateral circling movements when apomorphine was injected intraperitoneally, and ipsilateral circuling movements when amphetamine was injected.

The intraperitoneal injection of apomorphine (0.25mg base/kg) into rats, one week after the operation, was used as a reliable behavioural test

for successful lesioning. Rats that performed more than 200 contralateral turns within the first 25 min after a single dose of apomorphine were selected for the biochemical assays. Ungerstedt (8) demonstrated that degeneration of the nigrostriatal pathway occurred within 2 weeks after the intracerebral injection of 6-OH-DA. Histological examination of the brains from a number of animals injected with 6-OH-DA showed the production of a lesion in the desired position. Sections of the brain were cut in a cryostat and stained by the luxol fast blue-cresyl violet method (described in Section 5). On examination of the serial sections and by reference to the rat brain atlas of König and Klippel (140) it was found that the lesion was situated in the commencement of the nigrostriatal DAergic ascending pathway and it avoided the majority of the noradrenergic ascending axons (8).

2.2.2 Biochemical determinations

Rats were killed by decapitation and the brains quickly excised and placed in the ice-cold saline solution. Left and right sides of both striatum and substantia nigra were then carefully dissected out (as described in Section 1, 1.2) and the tissues frozen in liquid nitrogen within 2-3 min.

2.2.2a Determination of homovanillic acid (HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC)

The concentrations of (HVA) and (DOPAC) in the striatum were measured according to the gas chromatographic procedure described in the Appendix.

2.2.2b Measurement of γ-aminobutyric acid

The aminoacid γ-aminobutyric acid (GABA) was measured in the corpus striatum and the substantia nigra of the rat brain, with a sensitive gas chromatographic technique based on the method published by Pearson and Sharman (141).

The method involves homogenisation of the tissue, precipitation of proteins and isolation of GABA by passing the clear supernatant through a column filled with an Amberlite CG120 resin on which the GABA is retained, and elution of the aminoacid with ammonium hydroxide (NH_4OH). The alkaline eluate is evaporated to dryness and the residue is reacted with trifluoracetic anhydride and hexafluoroisopropanol. After evaporation to dryness under nitrogen, the residue is reacted with trifluoracetic anhydride and hexafluoroisopropanol. After evaporation to dryness under nitrogen, the residue is redissolved in ethyl acetate and injected into the gas chromatograph fitted with an electron capture detector.

The assay procedure is outlined below: Brain tissues dissected on ice as quickly as possible were immediately immersed in liquid nitrogen where they could be kept until the day of the assay (up to one month). For analyses, a tissue sample was weighed and homogenised in a 1.5 ml plastic centrifuge tube ('Eppendorf') with 800 µl of ice-cold 0.1 M HCl. The proteins were precipitated with 50 µl of concentrated (72% V/V) ice-cold perchloric acid (PCA) and following addition of a saturating amount of potassium chloride (KCl) the mixtures were centrifuged for 4 min at 14,000 g in a bench centrifuge ('Eppendorf'). The clear supernatant was transferred to a fresh tube. A portion, 25 µl, was diluted to 1 ml with cold 1 M HCl. (The remainder of the supernatant fluid was used for the assay of HVA and DOPAC). 0.5 ml of the acidified

supernatant was allowed to flow through a pasteur pipette filled to a depth of 2 cm with the Amberlite CG 120 cation exchange resin (100-120 mesh) (pretreated as described below) and fitted with cotton wool at one end to retain the resin. The column was washed with 10 ml of distilled water in 2 ml portions. The GABA was eluted from the column with 2 ml 2 M NH_4OH directly into a 3 ml reaction vial ('Reacti-vials', Pierce Co). The eluate was then frozen at -40°C (in a deep freeze) and freeze-dried (Jigtool freeze-drier) overnight. The dry residue was reacted at room temperature for 2 hours with 0.2 ml trifluoracetic anhydride (Aldrich Chem. Co.) and 0.1 ml hexafluoroisopropanol (BDH). After the 2 hours, the solution in the vial was evaporated to the point of dryness, at room temperature, under a stream of dry nitrogen and redissolved in 1 ml ethyl acetate (Reeve Angels - CT grade).

Samples of 2 µl were injected into the gas chromatograph with an automatic injector every 30 min, with column temperature 92°C and gas flow at 60 $ml/_{min}$ (corresponding to a pressure of 40 p.s.i.). The Hewlett-Packard model 7000 gas chromatograph was fitted with an electron capture detector arranged at 220°C for this assay. The carrier gas was a mixture of argon95%-methane 5%. The glass column was 9ft long, packed with 2% SE 52 coated on Diatomite CQ (100-200 mesh).

Before the application of the sample, the Amberlite resin was treated as follows: An amount of resin sufficient for several assays was suspended in 3 volumes of 2 M NH_4OH and stirred for 5 min. The NH_4OH was decanted and the resin was washed five times with distilled water by stirring and decantation and HCl (3 vol. 2M) was added to the resin and the suspension stirred for 5 min. The HCl was removed by

decantation and washing of the resin with 5 portions of distilled water. The acid treatment and water wash were repeated twice. After the last water wash a slurry of the resin in water was poured into a pasteur pipette, plugged with a small amount of cotton wool, to give a resin column of 2 cm. The column was treated by the passage, in succession, of 2 ml NH_4OH 2 M, 3 ml distilled water, 2 ml HCl 1 M and finally 10 ml distilled water. The acidified tissue extract was then applied to the column as already described.

Standard GABA solutions in NH_4OH 2 M were added into reaction vials at different concentrations (10-500 ng) and processed in parallel with the column eluates of tissue samples. Standard curves of peak area versus ng of standard GABA were made with all assays and the unknown samples determined accordingly. The peak area increased linearly in proportion to amounts between 10-500 ng (the linearity was not assessed beyond 500 ng). The recoveries of authentic GABA added to brain homogenates was 80 $\pm$ 7% (n = 14). Results were not corrected for recovery. The lowest limit of sensitivity of the method was about 1 ng.

2.2.2c Determination of glutamic acid decarboxylase activity

The activity of the enzyme glutamic acid decarboxylase (GAD) in the corpus striatum and the substantia nigra of the rat brain was measured with a combination of the radiochemical assay methods of Urquhart et al. (142) and Drummond and Phillips (143). The method involves the measurement of the amount of radioactive carbon dioxide evolved from L -[1-^{14}C] -glutamic acid on incubation with a tissue homogenate in the presence of pyridoxal phosphate, an essential cofactor of the enzyme. Labelled carbon dioxide is trapped in protozol, a strong base-solubilizer, and measured by liquid scintillation counting.

Assays were carried out within two days of obtaining brain samples which had been stored frozen. Homogenates (10 $\mu l/_{mg}$ tissue) of brain tissue samples were prepared in 1.5ml plastic centrifuge tubes (Eppendorf) using an electrically driven tissue homogeniser (Griffin and George) with teflon pestle (Jencons) in 1 mM potassium phosphate buffer, ice-cold, pH 6.5 (containing 0.1% $V/_V$ Triton X-100 and 0.1 mM pyridoxal-5-phosphate).

The incubation medium contained (final concentration) 100 mM potassium phosphate buffer pH 6.5, 0.5 mM pyridoxal-5-phosphate, 0.5mM dithiothreitol, 1.22 mM L -[1-^{14}C]-glutamic acid (specific activity 55 $mCi/_{mmol}$, Amersham), 0.5 mM unlabelled glutamic acid, 0.1 mM Triton X-100, 1mM sodium arsenite and distilled water (10% $V/_V$).

The homogenate (10 µl) and the incubation mixture (10 µl) were placed in an 1.5 ml plastic centrifuge tube (Eppendorf), which was in turn placed uncapped in a scintillation vial continaing 250 µl of protozol solubilizer. The scintillation vial was closed with a rubber seal ('Suba seal') to give a gas tight closure. After incubation accompanied by shaking for 20 min at 37°C in a water bath, the reaction was terminated by the injection of 100 µl 3 M H_2SO_4 into the plastic tube through the rubber seal. The vial was left in the water bath at 37°C for 1 hour, in order to allow complete absorption of the released $^{14}CO_2$ by the protozol. The Eppendorf tube was removed and the outside washed with 2 ml ethanol, the washing being added to the protozol. Toluene scintillant (10 ml) was then added to the fluid in the vial, the contents mixed and the vial was placed in the scintillation counter (Nuclear Chicago Mark II) and the radioactivity measured for 1 min.

GAD enzyme assays were carried out in duplicate. The counting efficiency was 68 per cent. Results of the enzyme assays were averaged and corrected for any radioactivity observed in assay blanks. Protein content of tissues was determined by the method of Lowry et al. (144) in duplicate 5 µl aliquots of the homogenates with bovine serum albumin as the standard, using a Gilford 250 spectrophotometer. The results were expressed as n moles $^{14}CO_2$ formed per mg of protein in the tissue sample per hour of incubation. Assay blanks, consisting of tissue homogenates (10 µl) without radioactive glutamate or of reaction mixtures without homogenates, were processed in parallel with the tissue samples. Both blanks gave about the same number of counts, which were subtracted from the counts obtained from the tissue samples in the calculation of results.

Measurement of counting efficiency

10 µl of a standard solution of toluene-$\left[^{14}C\text{-methyl}\right]$ with specific activity 1 µCi/ml was added in a vial, mixed with 10 ml of toluene scintillation fluid and counted for 1 min. Since 1 µCi $\equiv 2.22 \times 10^6$ dpm, 0.01 µCi $\equiv 2.22 \times 10^4 = 22{,}200$ dpm. The efficiency is the ratio: $\frac{\text{cpm (of the vial content)}}{22{,}200}$, (cpm: counts per min; dpm: decompositions per min.).

Calculation of results

The number of cpm obtained from each sample, after subtraction of the blank values, were corrected for the counting efficiency, the time of incubation, the amount of protein present in the aliquot of the homogenate used in every individual assay and the specific activity of the radioactive glutamate in the assay. The following formula was used for the calculation of the results:

$$(\text{cpm}-\text{"blank"cpm}) \times \frac{100}{\text{efficiency}} \times \frac{60\text{min}}{20\text{min}} \times \frac{1}{\text{mg}_{\text{protein}}/\text{mg}_{\text{tissue}}} \times \frac{1\text{nmole } {}^{14}CO_2}{\text{dpm liberated}/\text{nmole } {}^{14}CO_2} =$$

n moles $^{14}CO_2$ formed per mg of protein in the tissue sample per hour of incubation.

Reagents

Dithiothreitol or Cleland's reagent (DTT), from Koch-Light; Pyridoxal-5-phosphate (PLP) and L-glutamic acid (potassium salt) from Sigma Chem. Co.; Sodium arsenite from BDH; $\left(1-{}^{14}C\right)$-L-glutamic acid, specific activity 23 mCi/mmol, from Amersham Radiochemical Centre; Protozol, from New England Nuclear; 2,5-diphenyloxazol (PPO) and 1,4-di-[2-(4-methyl-5-phenyloxazolyl)-benzene] (POPOP), from Koch-Light. The toluene scintillation fluid contained 8.5 g PPO and 0.225 g POPOP in 2 l toluene.

All other reagents and solvents were of analytical grade.

2.2.2d Determination of choline acetyltransferase activity

The activity of the enzyme choline acetyltransferase (CAT) in rat brain areas (corpus striatum and substantia nigra) was measured with the rapid radiochemical method of Fonnum (145). The method involves measurement of the radioactive acetylcholine (Ach) formed from $\left(1-{}^{14}C\right)$-acetylcoenzyme A and excess choline, by an activated tissue preparation at a pH of 7.4, in the presence of eserine (acetylcholinesterase inhibitor) and a high concentration of NaCl. The labelled Ach formed is then isolated by liquid cation exchange using sodium tetraphenylboron (kalignost) in acetonitrile and toluene scintillation mixture, and the extraction is carried out directly in the scintillation vial.

Labelled Ach is then determined by liquid scintillation counting at high efficiency in the biphasic aqueous toluene scintillation solution mixture.

Homogenates (10 µl/mg tissue) of corpus striatum and substantia nigra were prepared in 1 mM EDTA ice-cold, pH 7.0 (containing 0.1 V/v Triton x-100 for the total activation of the enzyme). The tissue samples were homogenised in 1.5 ml plastic eppendorf tubes using an electrically driven tissue homogeniser (Griffin and George) with teflon pestle (Jencons). The homogenates were kept on ice until the incubation.

The incubation mixture contained (final concentration). 0.2 mM [1-^{14}C]-acetyl-CoA, 20 mM EDTA pH 7.4, 20 mM choline chloride, 600 mM NaCl, 100 mM sodium phosphate buffer pH 7.4, 0.2 mM eserine salicylate and 1 mg/ml bovine serum albumin. The labelled acetyl-CoA (specific activity 58 mCi/mmol) was mixed with the unlabelled compound to give a final concentration of 2 mM.

The tissue homogenate (10 µl) was placed in a stoppered 15 ml glass centrifuge tube with conical bottom and the incubation mixture (10 µl) was added. The solution was mixed and incubated with shaking for 15 min at 37°C in a water bath. At the end of the incubation, the tubes were placed on ice, and the reaction was terminated by the addition of 5 ml of ice-cold 10 mM sodium phosphate butter, pH 7.4, containing 250 µg of acetylcholine chloride. 2 ml of acetonitrile (containing 10 mg of kalignost) was then added to the tube and the mixture was shaken and transferred to a scintillation vial, followed by the addition of 10 ml of toluene scintillation fluid. The liquid in the vial was vortex mixed for 1 min, during which the Ach was extracted into the toluene phase, whereas the acetyl-CoA was left in

the aqueous phase. The scintillation vial was placed in the scintillation counter and the two layers were allowed to separate for 30 min. The samples were counted for ^{14}C for 10 min.

Enzyme assays were carried out in duplicate. The counting efficiency was 67 per cent. Results of the assays were averaged and corrected for any radioactivity observed in assay blanks. Protein content of tissues was determined by the method of Lowry et al.(144) in duplicate 5 µl aliquots of the homogenates, with bovine serum albumin as the standard, using a Gilford 250 spectrophotometer. The results were expressed as n moles 1-^{14}C-Ach formed per mg of protein per hour of incubation. The counting efficiency was measured as described for the GAD assay. The number of counts per min (cpm) obtained from each sample, after subtraction of the blank values, were corrected for the counting efficiency, the duration of the incubation, the amount of protein present in the portion of the homogenate used in every individual assay and the specific activity of the radioactive acetyl-CoA in the assay. Blank samples were prepared as in the GAD assay.

Reagents

[1-^{14}C]-acetyl-CoA specific activity 58 $mCi/_{mmol}$, from Amersham Radiochemical Centre;

acetyl-S-CoA and bovine serum albumin, from Sigma Chem. Co.;

choline chloride, acetylcholine chloride and eserine salicylate, from BDH;

2,5-diphenyloxazol (PPO) and 1,4-di-[2-(4-methyl-5-phenyloxazol)-benzene] (POPOP), from Koch-Light;

tetraphenylboron, sodium salt (kalignost) from Fluka.

The toluene scintillation fluid contained 8.5 g PPO and 0.225 g POPOP in 2 l of toluene.

All other reagents and solvents were of analytical grade.

2.2.2e Estimation of dopamine and noradrenaline in the striatum

The estimation of dopamine (DA) and noradrenaline (NA) in the striatum was carried out using a radiometric assay employing methylation of endogenous DA and NA by catechol-O-methyl transferase (COMT), with S-adenosyl-(methyl-^{3}H)-methionine as methyl donor, followed by extraction and measurement of the labelled 3-methoxytyramine and normetanephrine formed. The method used was derived from that described by Palkovits et al. (147). The assay procedure is outlined below:

Striatal tissue (about 20 mg) was homogenised in 0.1 M perchloric acid (300 µl/10 mg tissue). The homogenate was centrifuged at 10,000 g for 15 min and 300 µl of the supernatant fluid used for the subsequent assay. 300 µl aliquots of 0.1 M perchloric acid were used as blanks and standards of DA added to brain extracts were run with all assays.

Standards, samples and blanks were reacted with labelled S-adenosylmethionine in the presence of COMT. 300 µl of samples were added to a mixture containing 500 µg of dithiothreitol, 0.5 µmol $MgCl_2$, 140 µmol Tris-HCl buffer pH 9.6, 2.5 µl of rat liver COMT (prepared as described by Axelrod and Tomchick, (146) and 2.5 µCi of S-adenoxyl-(methyl-^{3}H)-methionine. After incubation at 37.5°C for 1 hour, the reaction was stopped by the addition of 500 µl 0.5M borate buffer, pH 10. After addition of non-radioactive carrier

(7 µg methoxytyramine and 7 µg normetanephrine) and 1 µg EDTA in a total volume of 50 µl, the O-methylated reaction products were extracted into 9 ml of water-saturated ethyl acetate-methanol (10:1 $V/_V$) by shaking for 30 sec. The phases were separated by centrifugation (1,000 g for 5 min) and 0.5 ml of the organic layer transferred to another tube containing 0.5 ml of 0.5 M borate buffer (pH 10). After vortex mixing for 15 sec and centrifuging for 5 min, 8 ml of the organic phase were transferred to another tube containing 0.5 ml of 0.1 M HCl into which the O-methylated products were extracted into the aqueous phase by mixing on a vortex for 30 sec. The tubes were centrifuged for 5 min and the organic phase was aspirated and discarded. The acid aqueous phase was washed with an additional 8 ml of water-saturated ethyl acetate by mixing for 15 sec and, after centrifugation, the ethyl acetate was aspirated and discarded.

The procedure so far described results in the methylation of both NA and DA and the extraction of their methylated derivatives. In order to separate normetanephrine (NA derivative) from methoxytyramine (DA derivative), the side-chain of normetanephrine is cleaved by metaperiodate at the β-hydroxyl position to form vanillin, which is then separated from methoxytyramine. To this aim, the acid aqueous phase described above was reacted with 50 µl of 3% ($w/_V$) sodium metaperiodate and 0.5 ml of 0.5 M sodium phosphate buffer (pH 7.5) for 3 min at 0°C. The reaction was stopped by the addition of 50 µl of 10% ($V/_V$) glycerol and 10 ml of toluene was added. After vortex mixing for 30 sec and centrifugation, the toluene layer was separated and used for NA estimation as it contained the labelled vanillin. The aqueous phase contained labelled

methoxytyramine and was saved for the DA determination. 5 ml of toluene was added to the aqueous layer and the mixture shaken and centrifuged to separate the two phases. 0.5 ml of 1 M borate buffer (pH 11), and 6 ml of toluene-isoamyl alcohol (3:2) was added to the aqueous layer. After mixing and centrifugation, 5 ml of the organic layer was taken and added to 10 ml of NE 260 liquid scintillation fluid. All samples were counted for 10 min.

To assay NA, the tubes containing 1 N NaOH and toluene (after the periodate cleavage reaction) were mixed, the organic phase removed and the aqueous phase containing the ^{3}H-methyl-vanillin was acidified with 0.1 ml of glacial acetic acid. 10 ml of toluene were added to each tube and the tubes were shaken for 30 sec. 9 ml of the organic phase were transferred to a scintillation vial containing 0.4 ml of Liquifluor (scintillation fluid) and counted for 10 min. Blanks and control samples containing known amounts of NA and DA were run in parallel with the unknown tissue samples.

Efficiency of radioactive counting

Efficiency of the counting of the tritiated products of the DA-NA assays was 30%. All samples were corrected for quenching by the external standard channels ratio method.

Reagents

Dithiothreitol or Cleland's reagent (DTT), from Koch-Light; S-adenosyl-(methyl-^{3}H)-methionine, specific activity 5-15 $Ci/_{mmol}$, from the Radiochemical Centre, Amersham; 3-methoxytyramine and normetanephrine, from Sigma; NE 260, from Nuclear Enterprises Ltd; Liquifluor, from New England Nuclear Corp.

2.2.2f Statistical analysis

The significance of the difference between two independent samples was determined by Student's t test. The differences between arithmetic means were evaluated by the Student's t test for unpaired data, whereas the difference between individual paired data was evaluated by the Student's t test for paired samples. The distribution of the results was sufficiently close to normal and the number of results in each group usually greater than 5, thus allowing the application of parametric statistics.

Results presented in the form:

$$\bar{X} \pm sd(n)$$

represent a mean ($\bar{X}$) plus or minus the standard deviation (sd) of n observations. A two-tailed Student's t test was routinely applied.

The confidence limit used in assessing statistical significance was that the probability (p) was less or equal to 0.05.

2.3 RESULTS

2.3.1 Dopamine metabolism in the corpus striatum after 6-hydroxydopamine lesions of the substantia nigra

Destruction of the catecholaminergic cell bodies in the substantia nigra with 6-OH-DA caused degeneration of the nigrostriatal DAergic pathway, including the axons and the nerve terminals in the corpus striatum. This was evident in the DA levels of the striatum, in the side ipsilateral to the lesion, which fell by 90% compared to the contralateral side (Table 2.1). This effect of the lesion, which was observed 1 - 2 months after the operation, confirmed similar results reported in the literature. Surprisingly, the concentrations of HVA and DOPAC in the ipsilateral striatum were only reduced by about 30% of those of the intact side (Table 2.1).

When the destruction of the nigral DAergic cell bodies was complete, as in another group of animals, the disappearance of DA from the ipsilateral striatum was almost total (98%), but the HVA and DOPAC concentrations were again reduced by only 40 and 43%, respectively, of those of the intact striatum (Table 2.1).

The lesion had no significant effect on the amine or on the metabolite concentrations in the contralateral striatum, these being not significantly different from those of control animals; the concentrations of DA or of the metabolites in the ipsilateral striatum were significantly lower than in the contralateral side.

Table 2.1

Effects of 6-OH-DA lesions of the left substantia nigra of rats on the concentrations of dopamine and its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in the corpus striatum.

	Dopamine μg/g tissue		HVA μg/g tissue			DOPAC μg/g tissue		
	Left	Right	Left	Right	Difference between sides (paired t test)	Left	Right	Difference between sides (paired t test)
Group 1	0.183 ± 0.03 (4) 2%	9.53 ± 1.74 (4)	0.61 ± 0.09 (4) 60%	1.01 ± 0.14 (4)	p<0.001	0.62 ± 0.11 (4) 57%	1.08 ± 0.17 (4)	p<0.001
Group 2	1.07 ± 0.14 (5) 9%	10.48 ± 2.07 (5)	0.77 ± 0.21 (10) 70%	1.15 ± 0.22 (10)	p<0.001	0.80 ± 0.17 (10) 68%	1.17 ± 0.20 (10)	p<0.001

Figures represent the mean ± standard deviation; number of animals is in parentheses.
Statistical significance was evaluated using Student's t test for paired data.
Groups 1 and 2 represent lesioned animals with a complete or a non-complete lesion, respectively.

Percentages = mean amount found in the striatum ipsilateral to the lesioned substantia nigra as a percentage of mean amount found in the contralateral striatum.

2.3.2 Effect of treatment with apomorphine or haloperidol on dopamine metabolism

Apomorphine, administered at a dose of 1 mg/kg i.p. 20 min before decapitation of the animals, produced in the striatum contralateral to the lesion a significant decrease in both HVA and DOPAC ($p < 0.05$), but it failed to alter the levels of the two metabolites in the ipsilateral striatum, compared to the same side in lesioned animals injected with saline solution (Table 2.2).

Haloperidol, given at a dose of 1 mg/kg i.p. 30 min before killing caused a significant rise to 247 and 245% in HVA and DOPAC, respectively, in the striatum ipsilateral to the lesion compared to the same side in saline treated rats. The concentrations of HVA and DOPAC in the striatum contralateral to the lesion were 329 and 333%, respectively, of those in the same side of saline-treated rats (Table 2.2).

After treatment with haloperidol the concentrations of HVA and DOPAC in the striatum ipsilateral to the lesion were significantly lower than the concentrations in the contralateral side, i.e. 50% of the contralateral side. On the contrary, after apomorphine there was no significant difference between the levels of the metabolites of the two sides (Table 2.2).

2.3.3 Changes in GABA levels of the striatum and the substantia nigra

The concentration of GABA in the denervated side of the striatum (Table 2.3) was significantly higher than in the intact side ($p < 0.001$, paired t test). Treatment of the animals with apomorphine at a dose of 1 mg/kg i.p. 30 min before killing led to a significant increase of GABA in the striatum contralateral to the lesion,

Table 2.2

Concentration of HVA and DOPAC in the corpus striatum of rats with a unilateral lesion in the left side of the substantia nigra, and the effect of apomorphine and haloperidol on the levels of these metabolites.

	HVA µg/g tissue			DOPAC µg/g tissue		
Treatment	Left	Right	Difference between sides (paired t test)	Left	Right	Difference between sides (paired t test)
No lesion	1.08 ± 0.13 (5)	1.12 ± 0.08 (5)	n.s.	1.15 ± 0.18 (5)	1.19 ± 0.21 (5)	n.s.
Lesion + saline	*2 0.77 ± 0.21 (10)	1.15 ± 0.22 (10)	$p < 0.005$	*2 0.80 ± 0.17 (10)	1.17 ± 0.20 (10)	$p < 0.001$
Lesion + apomorphine	*2 0.73 ± 0.21 (6) 95%	*3,*6 0.72 ± 0.14 (6) 63%	n.s.	*1 0.82 ± 0.15 (5) 102%	*1,*5 0.85 ± 0.20 (5) 73%	n.s.
Lesion + haloperidol	*2,*6 1.90 ± 0.53 247% (6)	*4,*7 3.78 ± 0.23 329% (6)	$p < 0.001$	*2,*6 1.96 ± 0.44 245% (6)	*4,*7 3.90 ± 0.47 333% (6)	$p < 0.001$

Legend for Table 2.2

Saline-treated lesioned animals serve as controls.

The rats were killed 20 min after apomorphine (1 mg/kg i.p.) and 60 min after haloperidol (1 mg/kg i.p.)

Figures represent the mean ± standard deviation; number of animals is in parentheses.

Statistical significance was evaluated using Student's t test for paired or for unpaired data.

Percentages = mean amount found as a percentage of mean amount found in the same side of the striatum in lesioned animals treated with saline solution.

*1, *2, *3, *4: significantly different from the same side in saline-treated lesioned animals ($p<0.05$, $p<0.01$, $p<0.0025$, $p<0.0005$, respectively)

*5, *6, *7: significantly different from the same side in unlesioned animals ($p<0.05$, $p<0.0025$, $p<0.0005$, respectively)

Table 2.3

Effects of apomorphine and haloperidol on the choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) activities and on GABA concentration in the two sides of the corpus striatum after 6-OH-DA lesions of the left side of the substantia nigra, or after sham-operations.

	CAT n moles ^{3}H-Ach formed/mg protein/hr			GAD n moles ^{14}C-CO_2 formed /mg protein/hr			GABA $\mu g/g$ tissue		
Treatment	Left	Right	Difference between sides (paired t test)	Left	Right	Difference between sides (paired t test)	Left	Right	Difference between sides (paired t test)
Lesion + saline	20.44 ± 2.19 (12)	15.48 ± 1.64 (12)	$p < 0.001$	51.18 ± 6.37 (10)	42.01 ± 6.94 (10)	$p < 0.05$	270 ± 61 (11)	138 ± 23 (11)	$p < 0.001$
Lesion + apomorphine	19.66 ± 3.29 (6)	14.56 ± 3.21 (6)	$p < 0.001$	48.00 ± 9.20 (6)	43.02 ± 7.20 (6)	n.s.	228 ± 88 (4)	232 ± 49* (4)	n.s.
Lesion + haloperidol	20.95 ± 2.51 (11)	17.93 ± 2.47* (11)	$p < 0.001$	47.45 ± 4.98 (5)	30.10 ± 6.66** (5)	$p < 0.01$	172 ± 44** (6)	104 ± 27 (6)	$p < 0.01$
Sham-operation + saline	16.66 ± 1.60 (3)	16.07 ± 2.31 (3)	n.s.	43.62 ± 8.98 (3)	42.49 ± 4.78 (3)	n.s.			
Sham-operation + apomorphine	16.85 ± 2.08	15.92 ± 2.34	n.s.	40.77 ± 5.40	40.17 ± 2.81	n.s.			
Sham operation + haloperidol	18.64 ± 2.23 (3)	17.60 ± 0.81 (3)	n.s.	30.98 ± 5.02 (3)	32.77 ± 3.50 (3)	n.s.			

Legend for Table 2.3

Saline-treated lesioned animals serve as controls.

Figures represent the mean ± standard deviation; number of animals is in parentheses.

Enzyme activities and GABA levels were measured about 40 days after the lesion.

Apomorphine (2 mg/kg i.p.) or haloperidol (1 mg/kg i.p.) were administered 30 min or 1 hour before killing, respectively.

Statistical significance was evaluated using Student's t test for paired or for unpaired data.

* $p < 0.01$ compared to the same side in saline-treated animals (Student's t test).

** $p < 0.05$ compared to the same side in saline-treated animals (Student's t test).

n.s: not statistically significant difference.

compared to the same side in lesioned animals treated only with saline ($p < 0.05$), but had no significant effect on the denervated side compared to the same side of lesioned animals treated with saline. Thus, the balance between the two sides with regard to GABA seems to have been restored, as was found with the levels of HVA and DOPAC after apomorphine administration (Table 2.2).

Treatment of rats with haloperidol (1 $mg/_{kg}$ i.p.) 60 min before killing, produced no significant effect on the GABA concentration of the intact side of the striatum compared to control, whereas a significant reduction was obtained in the denervated striatum compared to the same side in saline treated animals ($p < 0.05$). The concentration in the denervated side was significantly lower than in the intact side following the administration of haloperidol ($p < 0.01$, paired t test).

In the substantia nigra (Table 2.4), the lesion produced a significant reduction of GABA concentration in the destroyed left side compared to the right side which was not affected by the 6-OH-DA injection ($p < 0.01$, paired t test). Administration of apomorphine (1 $mg/_{kg}$ i.p.) to successfully lesioned animals, 30 min before decapitation, caused no significant effect on GABA in the intact side of the substantia nigra compared to the same side in lesioned animals treated with saline, but the reduction in the lesioned side was restored by a significant increase of the GABA levels in this side, compared to the lesioned side in animals treated with saline ($p < 0.01$). A significant difference was found between the lesioned and the intact side in rats treated with apomorphine ($p < 0.01$, paired t test).

Table 2.4

Effects of apomorphine and haloperidol on the choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) activities and on GABA concentration in the two sides of the substantia nigra after 6-OH-DA lesions of the left side of this structure.

	CAT n moles ^{3}H-Ach formed/mg protein/h			GAD n moles ^{14}C-CO_2 formed/mg protein/h			GABA µg/g tissue		
Treatment	Left	Right	Difference between sides (paired t test)	Left	Right	Difference between sides (paired t test)	Left	Right	Difference between sides (paired t test)
Lesion + saline	5.50 ± 0.92 (12)	7.05 ± 0.95 (12)	$p < 0.005$	58.22 ± 7.21 (6)	67.60 ± 5.94 (6)	$p < 0.05$	298 ± 63 (13)	443 ± 95 (13)	$p < 0.01$
Lesion + apomorphine	3.83 ± 1.31** (6)	5.44 ± 1.26** (6)	$p < 0.05$	58.99 ± 5.51 (5)	83.26 ± 13.22* (5)	$p < 0.005$	493 ± 84* (8)	385 ± 83 (8)	$p < 0.01$
Lesion + haloperidol	5.14 ± 1.06 (5)	7.49 ± 1.58 (5)	$p < 0.05$	83.90 ± 11.19* (6)	68.05 ± 12.20 (6)	$p < 0.05$	410 ± 79** (6)	455 ± 76 (6)	n.s.
Sham-operation + saline	7.42 ± 1.46 (3)	6.11 ± 0.60 (3)	n.s.	71.39 ± 14.39 (3)	72.11 ± 11.56 (3)	n.s.			
Sham-operation + apomorphine	5.67 ± 0.61 (4)	5.17 ± 1.10 (4)	n.s.	75.28 ± 7.48 (4)	83.97 ± 11.23 (4)	n.s.			
Sham-operation + haloperidol	6.86 ± 0.35 (3)	6.66 ± 0.55 (3)	n.s.	63.14 ± 11.66 (3)	64.46 ± 3.82 (3)	n.s.			

Legend for Table 2.4

Saline treated lesioned animals serve as controls.

Figures represent the mean ± standard deviation; number of animals is in parentheses.

Enzyme activities and GABA levels were measured about 40 days after the lesion.

Amomorphine (2 mg/kg i.p.) or haloperidol (1 mg/kg i.p.) were administered 30 min or 60 min before killing, respectively.

Statistical significance was evaluated using Student's t test for paired or for unpaired data.

* $p < 0.01$ compared to the same side in saline treated animals (unpaired data)

** $p < 0.05$ compared to the same side in saline treated animals (unpaired data)

n.s: not significant difference

Administration of haloperidol (1 mg/kg i.p.), 60 min before decapitation, caused no significant effect on GABA concentration in the intact side of the substantia nigra compared to the same side in animals treated with saline, but it restored the balance between the two sides by a significant increase of the GABA levels in the lesioned side compared to the same side in animals treated with saline ($p < 0.05$).

2.3.4 Lesion-induced changes in enzyme activities (Fig. 2.1.)

Choline acetyltransferase (CAT) (Tables 2.3 and 2.4.)

6-hydroxydopamine lesions successfully placed in the substantia nigra caused a significant fall in the activity of the enzyme CAT in the lesioned side compared to the intact side of this region. In the corpus striatum a significant rise of the enzyme was observed in the ipsilateral compared to the side contralateral to the lesion ($p < 0.005$ and $p < 0.001$, paired t test, respectively).

Administration of apomorphine at a dose of 2 mg/kg i.p., 30 min before killing, caused no significant effect on either side of the striatum, the side ipsilateral to the lesion still having higher CAT activity compared to the contralateral side ($p < 0.001$, paired t test). In the substantia nigra, apomorphine produced a further decrease in the lesioned side, and the imbalance of CAT between the two sides remained ($p < 0.05$, paired t test).

Administration of haloperidol (1 mg/kg i.p.), 1 hour before decapitation, produced no significant effect on CAT activity of either side of the substantia nigra, and, therefore, the lesioned side was still lower than the intact side with regard to the CAT enzymatic activity ($p < 0.05$, paired t test). In the striatum, haloperidol did not alter the lesion-induced imbalance; the

ipsilateral side was still higher than the side contralateral to the lesion ($p < 0.001$).

Groups of sham-operated animals were subjected to the same treatment (i.e. saline, apomorphine, haloperidol) and showed no imbalance in CAT between the two sides of the striatum or the substantia nigra.

Glutamic acid decarboxylase (GAD) (Tables 2.3 and 2.4)

Similarly to CAT, GAD activity was higher in the ipsilateral compared to the contralateral striatum ($p < 0.05$, paired t test) in successfully lesioned animals, but it was significantly lower in the lesioned compared to the intact side of the substantia nigra ($p < 0.05$, paired t test).

Apomorphine (2 mg/kg i.p.), given 1 hour before killing, had a stimulating effect on the activity of GAD in the intact side of the substantia nigra ($p < 0.01$) but no significant effect on the activity in the lesioned side, so that an imbalance between the two sides was produced (lesioned side lower than the intact side) ($p < 0.005$, paired t test).

Haloperidol (1 mg/kg i.p.), given 1 hour before killing, had no significant effect on the GAD in the intact side, but it caused a marked increase of GAD in the lesioned side of the substantia nigra, which thus became significantly higher than the intact side ($p < 0.05$, paired t test) and than the lesioned side of saline-treated animals ($p < 0.005$).

In the striatum, apomorphine had no significant effect on either side's GAD activity compared to the same side of saline-treated lesioned animals, but the imbalance between the two sides with regard to the GAD was eliminated to the extent that no significant difference was any longer observed between them. In the same region,

haloperidol produced a significant reduction of GAD activity in the side contralateral to the lesion but had no significant effect on the enzyme activity in the side ipsilateral to the lesion, thus leading to an imbalance similar to that observed in saline-treated animals.

Sham-operated animals, treated with saline or apomorphine or haloperidol showed no difference in GAD between the two sides in the striatum or the substantia nigra.

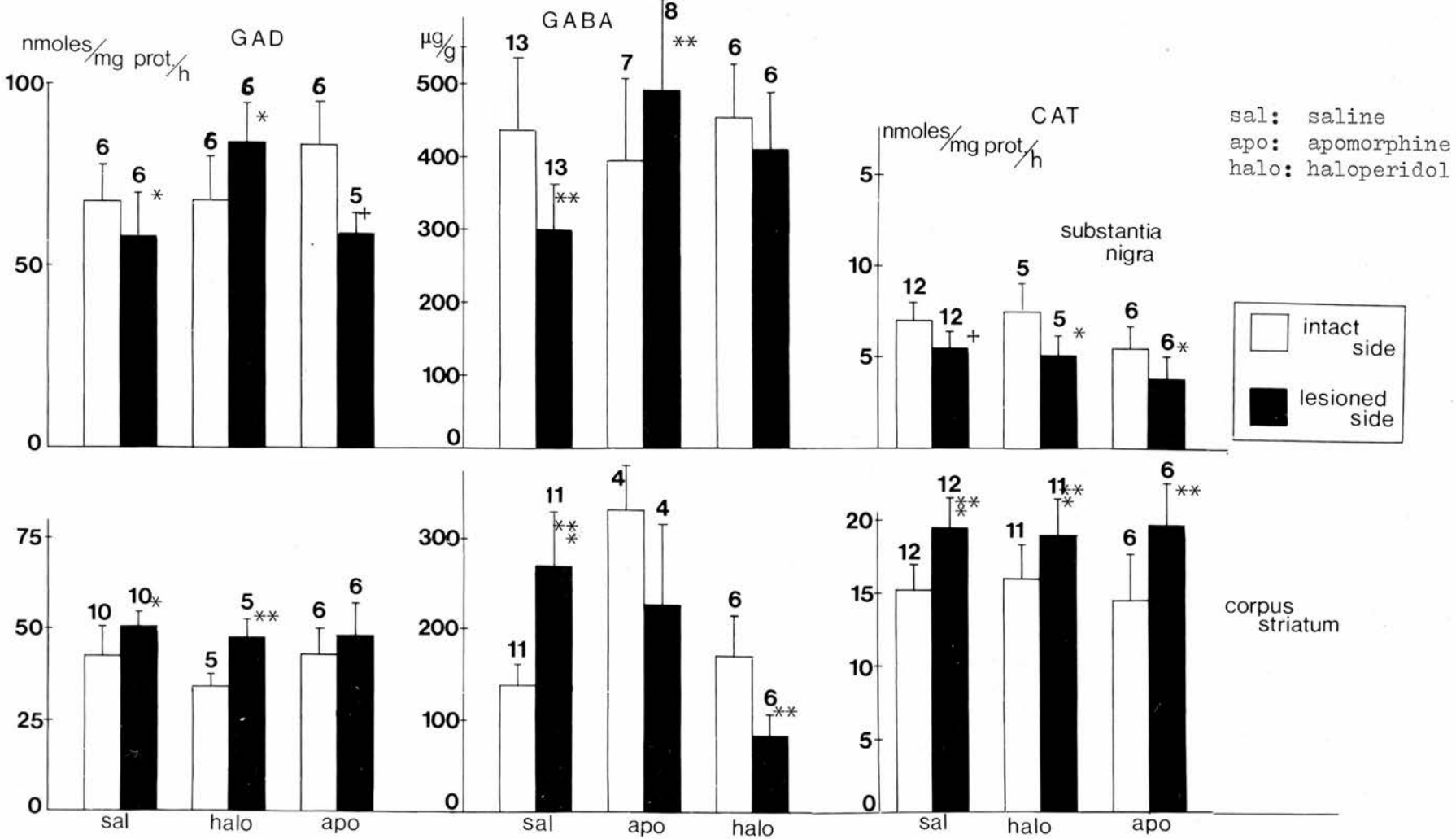


Fig. 2.1.

Effects of 6-OH-DA lesions in the substantia nigra of the rat brain on GAD, GABA and CAT in the substantia nigra and the corpus striatum

Statistical significance (paired t test, compared to the other side)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; + $p < 0.005$

2.4 DISCUSSION

2.4.1 The primary effect of the lesion: degeneration of the dopaminergic pathway

It is well known that lesions of the substantia nigra, which contains the cell bodies of DAergic neurons, innervating the striatum, cause orthograde degeneration of the axons and nerve terminals of these neurons (8). This is reflected in a significant depletion of DA from the denervated striatum (107). Due to the high specificity of the 6-OH-DA-induced lesion (149), the deviation in movements and posture of the animal towards the lesioned side (8,107) is in all probability an expression of the imbalance in DA transmission induced by the lesion. Total DA loss in the striatum is not usually achieved by the nigral 6-OH-DA lesion; the extent of the lesion (loss of cells from the substantia nigra) determines the extent of DA loss in the ipsilateral striatum in the case of almost complete lesions (150).

In the present study, the changes in the DA levels of the striatum were found to be comparable to those reported in the literature. Lesions of the substantia nigra produced, in separate groups of animals, reduction of DA concentrations by 98 and by 90 per cent. Since the substantia nigra seems to be the exclusive source of striatal DAergic innervation (8) the reduction of the DA levels in the striatum probably mirrors the extent of the lesion damage, unless compensatory changes occur. Increased activity of the remaining intact neurons in the lesioned side would result in increase in the synthesis and utilisation of DA. Indeed, an increase in DA synthesis and turnover was found by Agid

et al.(123) to result from a partial degeneration of the nigrostriatal DAergic pathway, probably mediated through an unidentified feedback process. The same process may be responsible for the discrepancy observed in the present study between the changes in striatal DA concentrations and in the striatal concentrations of its main metabolites HVA and DOPAC. Thus, disappearance of 98 per cent of DA in the denervated striatum was accompanied by a fall in HVA and DOPAC concentrations of only about 40 per cent. Similarly, a lesion which caused a 90 per cent reduction in striatal DA levels (compared to the side contralateral to the lesion) produced only a 30 per cent reduction in HVA and DOPAC (Tables 2.1 and 2.2). A hyperactivity of the remaining intact neurons, induced, perhaps, by the DA deficiency at some postsynaptic receptor sites, and mediated through an unknown feedback mechanism, probably induced a markedly accelerated turnover of DA in the partially denervated side.

The finding that the extent of DA deficiency is not proportional to the extent of the deficiency in the metabolite concentrations, may indicate that a large number of remaining intact neurons are hyperactive, and that this number may be inversely correlated to the extent of the damage. This interpretation is in agreement with the demonstrated correlation between changes in NAergic neuronal activity and NA turnover, both in central (27) and peripheral (26) neurons. The central DAergic neurons also seem to behave in the same way, but a paradoxical increase of DA synthesis as a result of tyrosine hydroxylase activation follows cessation of impulse flow (25). The latter phenomenon, which occurs after lesions of the nigrostriatal DAergic pathway, could explain the finding of smaller percentage decrease in the concentrations of the

metabolites of DA than in the concentrations of the amine. A reduction of DA at the terminal area may result in a decrease of the end-product inhibitory effect of DA on tyrosine hydroxylase, thus leading to increased synthesis and turnover.

2.4.2 Pre-and postsynaptic effects of apomorphine and haloperidol

The direct DA receptor stimulant apomorphine (109) had no significant effect on DA metabolite concentrations in the denervated striatum, although it caused a significant reduction in the striatum contralateral to the lesion. Thus, the lesion-induced difference between the two sides of the striatum with regard to HVA and DOPAC disappeared when the rats were treated with apomorphine; the two sides seemed to have balanced concentrations of the two metabolites, indicating equal turnover or metabolism. The destruction of the majority of the fibres of the nigrostriatal DAergic pathway appeared to prevent the reduction of DA turnover that probably follows stimulation of DA receptors by apomorphine.

The presence of presynaptic DA receptors, which are involved in the control of the rate of DA synthesis in the terminals of DA-containing neurons has been demonstrated (63). The presence of postsynaptic DA receptors, probably located on cholinergic or GABAergic neurons of the striatum (94,127) is well established.

Supersensitivity of postsynaptic DA receptors in the striatum following lesions of the nigrostriatal pathway, manifested either in increased number of DA receptor sites (119) or in increased activity of DA-sensitive adenylate cyclase (116), or in the well known behavioural supersensitivity (8), does not seem to be reflected in a change in DA turnover following DA receptor stimulation. If the 'supersensitive' DA

receptors were mediating the effects of apomorphine on DA turnover, an increased effect would be seen in the denervated 'supersensitive'side. The lack of effect of apomorphine on DA metabolite levels in the denervated striatum would either indicate a subsensitivity of the presynaptic DA receptor population thought to be located on the remaining intact neurons, or inactivation of the mechanism by which DA receptor stimulation results in decreased DA turnover in the intact striatum.

The finding that the lesion did not markedly prevent the effect of the DA receptor blocker haloperidol on striatal HVA and DOPAC does not exclude the possibility that the postsynaptic 'supersensitive' DA receptors mediate the effect of this drug on DA turnover. The blockade of the 'supersensitive' postsynaptic DA receptors in the striatum by haloperidol does not appear to produce a 'supersensitive' response with reference to DA metabolism. Additive effects may occur. The small number of intact DAergic neurons may over-react in compensation for the extensive denervation, and the effect of haloperidol is an increased activity of the DAergic neurons. The unknown compensatory mechanisms, induced by interruption of DA neurotransmission (by haloperidol) and by the lesion, might be of similar nature.

In the light of the observations made by Seeman and Lee(163), that with electrically stimulated slices of brain tissue the stimulus-induced release of DA was reduced by neuroleptic drugs (such as haloperidol), whereas the spontaneous efflux of the catecholamines was increased, it was suggested that neuroleptic drugs inhibit the coupling between the nerve impulse and DA release from the nerve ending. The results of the present experiments suggest, however, that the contribution of this mechanism to the neuroleptic-induced increase in the striatal levels of

HVA and DOPAC is a minor one. As Table 2.2 shows, i.p. administration of haloperidol to rats lesioned in the substantia nigra with 6-OH-DA, produced a 250% increase of the metabolite concentrations in the ipsilateral striatum, whereas in the contralateral side the increase was not markedly higher, i.e. only 330%. Thus, the lesion and the consequent destruction of the vast majority of the ascending DAergic fibres containing the presynaptic DA receptor sites, prevented the effect of haloperidol on DA metabolism by only a small degree. This supports the predominance of postsynaptic effects of haloperidol on DA turnover, compared to the presynaptic ones. On the contrary, stimulation of the presynaptic DA receptors seems to mediate the effect of apomorphine on striatal DA metabolism.

The integrity of the nigrostriatal DAergic pathway, which appears to be critical for the effect of apomorphine on striatal DA metabolism, does not seem to be necessary for the increase in striatal DA turnover induced by haloperidol. The exact mechanism or site of this action of neuroleptics is not clear, yet. Although destruction of the nigrostriatal DAergic fibres reduced to some limited extent the effect of neuroleptic agents on DA-containing nerve terminals in the striatum, as the present and previous studies by Bedard and Larochelle (164) have shown, destruction of the descending striatonigral fibres did not suppress the accelerated DA turnover in the striatum following haloperidol treatment (164, 165). Thus, the activation of DA-containing neurons in the striatum in response to neuroleptic drugs does not seem to depend significantly on the integrity of the known nigrostriatal or striatonigral fibres. Therefore, other mechanisms must be responsible for the observed increase in striatal DA turnover. A local phenomenon or other unknown neuronal

pathways might be involved in this effect. The postsynaptic DA receptor blockade seems to be the most probable process, activating as a result the DA turnover in the striatum by a local feedback mechanism, as suggested by Kehr et al.(63).

Recent reports concerning the site of action of DA receptor blockers and DA receptor agonists eliminate the number of possibilities for the regulation of DA metabolism in the striatum. Thus, the complete loss of striatal adenylate cyclase after intrastriatal injection of kainic acid, thought to be accompanied by destruction of postsynaptic DA receptors (166) did not prevent the effect of these drugs on DA metabolism. Consequently, these results indicate that an action at the level of the postsynaptic DA receptors is not a pre-requisite for the in vivo effects of DA receptor blockers and stimulants on DA metabolism. Location of the mechanism which exerts an inhibitory influence on DAergic neurons at a prejunctional level, being mediated by DA released from DAergic nerve terminals onto presynaptic DA receptors regulating tyrosine hydroxylase (168) or DA release (167) might explain the lack of effect of DA receptor stimulation on HVA and DOPAC in the denervated striatum, but cannot explain the increase in striatal metabolite levels by 250 per cent, induced by haloperidol in the denervated striatum (demonstrated in the present study).

Alternatively, assuming that there is hyperactivity of the remaining neurons, then, presumably, any presynaptic feedback inhibition of the release of DA is working maximally and, therefore, one might expect direct DA receptor agonists (e.g. apomorphine) not to affect the release of (and consequently the metabolite concentrations of) DA in the denervated striatum by acting on presynaptic DA receptors (as it would

in the intact side). However, receptor blockers (e.g. haloperidol) can still show a presynaptic action, preventing the presynaptic self-inhibition of DA release. Therefore, they cause an increase in DA release and consequently an increase in the concentrations of the metabolites in the denervated as well as in the intact striatum.

The results of the present study also do not support the location of the mechanism of regulation of striatal DA metabolism in the substantia nigra and the idea that it is mediated by DA released from DAergic dendrites on to DA receptors (autoreceptors) thought to be located on nigral DAergic neurons (53). The results of Garcia-Munoz et al.(165) also seem to exclude the possibility of a post-junctional location of this mechanism, mediated through a GABAergic striatonigral neuronal feedback loop.

Thus, local regulatory mechanisms which function in both corpus striatum and substantia nigra could be postulated to exist. The involvement of GABA or 5-HT inputs to the substantia nigra, and the known synaptic contacts of these neurons with the DAergic neurons in this region (73), combined with the known inhibitory effects of local application of these substances on cell firing (64) may be part of the postulated process of local dendritic control of DA release. The existence of a similar mechanism at the DAergic nerve terminal area of the striatum remains to be evaluated, but we could hypothesise that there is a local feedback system which connects the postsynaptic with the presynaptic sites of the DAergic neurons at the striatal level. Subsequent experiments seem to support this concept.

It has been suggested by Kehr et al(63) that receptor-mediated feedback mechanisms exist, which control the rate of synthesis and

turnover of DA in the striatum. These investigators suggested that the synthesis rate can be influenced by DA receptor stimulation or blockade, even in the absence of impulse flow, but that the turnover rate seems to depend to a considerable extent on impulse flow, as it was proposed by earlier work (169). The different experimental conditions might explain the disagreement of part of the above findings with the results of the present study. The fact that the above mentioned workers measured the DA synthesis and turnover immediately after the complete section of the nigrostriatal DAergic pathway contrasts with the present study, where the animals were sacrificed 1-2 months after a, probably, not complete lesion of the same pathway and by which time the compensatory processes may be activated.

Other more complex processes might occur in the denervated striatum in view of the fact that part of the DAergic nigrostriatal fibres seem to be intact and, probably, hyperactive, compared to the fibres in the contra-lateral side. The alteration of DA receptor sensitivity by the lesion (121) and, probably, the increase of the number of DA receptor sites in the lesioned striatum (119), make the deduction of any concrete conclusions from the study of the effect of haloperidol on DA metabolism difficult. The proposed complex modes of action of this agent on DA metabolism allow several interpretations of the findings.

2.4.3 The striatal dopamine-acetylcholine functional link

The complexity of the effects of the lesion would be expected, in view of the known synaptic contacts of DAergic nerve terminals with cholinergic neurons in the striatum (93,103). The cells of these striatal cholinergic neurons appear to receive an inhibitory influence from the nigrostriatal pathway (90,91,92). Postsynaptic DA receptors, probably

located on these neurons, mediate this influence. Therefore, changes in the sensitivity (121) or the number (119) of the striatal DA receptors (both phenomena seemingly resulting from the lesion of the nigrostriatal DAergic pathway) could exert an indirect effect on the functional state of the striatal cholinergic neurons. Interruption of the inhibitory DAergic fibres would largely release the cholinergic neurons from the inhibitory influence they receive and would be expected to result in neuronal hyperactivity.

This may be the mechanism underlying the finding that lesions of the substantia nigra produced in the denervated striatum a significant increase of choline acetyltransferase activity (CAT) compared to the contralateral side.

The enzyme CAT, synthesizing the neurotransmitter Ach, was chosen for examination, because, in addition to its role in catalysing the synthesis of the transmitter, which makes it a good marker of the activity of the cholinergic neuron, it also represents a specific protein produced by identifiable nerve cells. Long term effects of treatments, such as the 6-OH-DA lesion, might be manifested in an increase in enzyme activity secondary to an increase in enzyme-protein synthesis. Changes in protein turnover in the reactive cells might be reflected by changes in the accumulation in response to surgical manipulations of the nigrostriatal pathway. The increased biosynthesis of CAT in the denervated striatum suggests a rearrangement of priorities in enzyme biosynthesis favouring this enzyme, perhaps at the expense of the enzymes related to DA synthesis or turnover. Otherwise, it could result from a compensatory increase in biosynthesis of CAT presynaptically, in cholinergic neurons impinging on the intact and hyperactive DAergic neurons of the

denervated striatum, or at a postsynaptic level in the target neurons deprived of their normal DA innervation. These arguments are, however, weakened by the finding that the opposite pattern of change in CAT biosynthesis seems to occur during axonal injury: the proteins required for reconstitution of the axonal surface accumulate at the expense of those required for neurotransmission (170) and the accumulation correlates with the regenerative sprouting of the neurons (171).

The increased enzyme activity may well be mediated through an ionic or electrical mechanism. Such a mechanism could act through local changes in ion concentrations near the nerve-ending membrane. Depolarising agents such as ouabain or potassium ions have been found to increase the activites of tyrosine hydroxylase in the vas deferens (158) and adenylate cyclase in the brain (159). The 'supersensitive' DA receptors in the denervated striatum, probably located on cholinergic neurons, may modulate the activity of the enzyme CAT through membrane hyperpolarisation or depolarisation. Similarly, these receptors may alter the fluxes of specific ions (e.g. CA^{++}) critical for the activation of the neurotransmitter-synthesising enzymes present in the same neurons. Alternatively, there might be a direct allosteric interaction between the enzyme and molecules in the nerve ending membrane at the site of transmitter release.

Whatever the mechanism of the lesion-induced dynamic changes in enzyme biosynthesis might be, their functional importance could be assessed by considering the proposed cholinergic link mechanism at the axon terminals of the nigrostriatal DAergic neurons (172). In this formulation, impulse-induced presynaptic release of Ach from cholinergic neurons impinging on DAergic nerve terminals in the striatum would

act upon postsynaptic cholinergic receptors (located on DAergic terminals) to facilitate the subsequent release of DA. The enzyme acetylcholinesterase would be present in the vicinity of DA-containing terminals to inactivate the initially released Ach. Compatible with the hypothesis that Ach is a presynaptic neuromodulator of striatal DAergic nerve endings are the observations of Georguief et al.(174), who found that Ach at concentrations of 10^{-6} and 10^{-5} M enhanced the release of ^{3}H-DA from cat caudate nucleus and rat striatal slices. This scheme of neuronal interrelation is depicted in Fig. 2.2.

The data from the present experiments do not exclude prejunctional contacts of the two neuronal systems but totally discount the possibility that the lesions affect directly cholinergic neurons in the striatum, since lesion and degeneration of these neurons would be expected to reduce the enzyme activity or concentration. On the contrary, the increased CAT activity (and, probably, concentration) would result from an indirect effect of the lesion, and is indicative of increased activity of cholinergic neurons. Both Ach and CAT appear to be present within intrinsic striatal neurons (interneurons), since lesions destroying the majority of afferent pathways to this region do not cause a reduction of either Ach content or the CAT activity of the striatum (97,175).

It has been demonstrated that certain striatal neurons respond in an opposite fashion to iontophoretic application of Ach and DA: Ach enhances the firing of cells in the striatum, whereas DA depresses the majority of these cells (176). Therefore, it seems possible that some striatal cells could receive antagonistic neuronal inputs, i.e. excitatory cholinergic and inhibitory dopaminergic input. These antagonistic inputs have been hypothesized to constitute the functional balance which

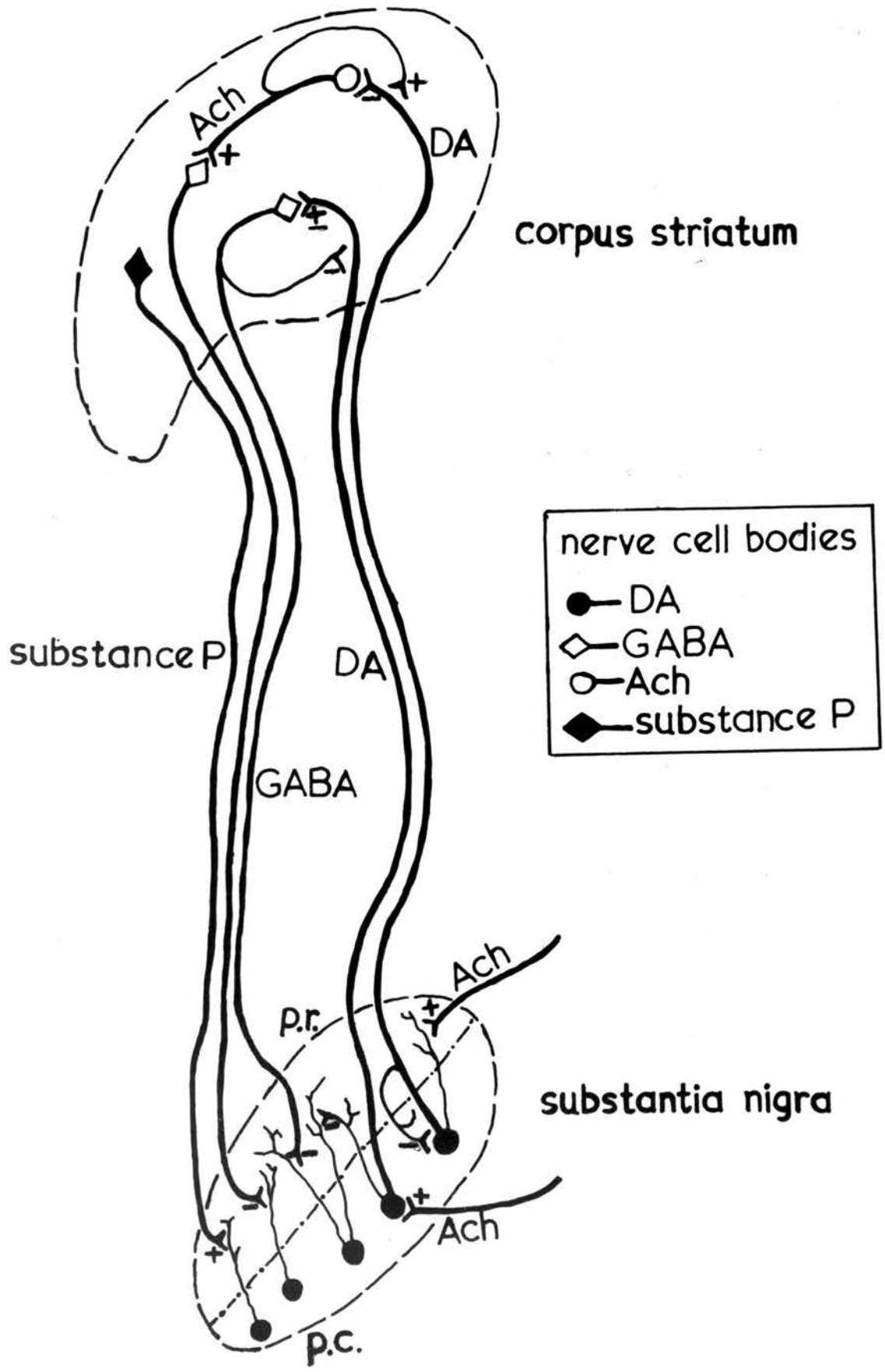


Fig. 2.2

A diagrammatic representation of some neuronal connections between the corpus striatum and the substantia nigra.

The signs + or - indicate excitatory or inhibitory, respectively, influence of the released transmitter on the postsynaptic neuron.

p.r. - pars reticulata
p.c. - pars compacta

determines the efferent neuronal outflow from the striatum, such as the striatonigral GABA-containing neurons (177).

Using various pharmacological and lesioning techniques, many investigators have noted that the activity of the striatal cholinergic system appears to be inversely correlated with the activity of the DAergic system, e.g. an increase in DAergic activity appears to coincide with a decrease in cholinergic activity and vice-versa (124,125). Lesions of the medial forebrain bundle, which destroyed the nigrostriatal DAergic neurons produced a decrease in the striatal steady-state levels of Ach and a postulated increase in striatal cholinergic neuronal activity (181). The present study, using a different approach, confirmed this hypothesis. Lesions of the nigrostriatal pathway were shown to lead to a long-lasting or, possibly, permanent increase of CAT activity in the denervated striatum.

Additional experimental evidence supports the concept of a cholinergic-DAergic balance in the striatum. Experiments by Sourkes and Poirier (178) demonstrated that a reduction of neuronal activity in the nigrostriatal pathway produced by either placing lesions (i.e. a procedure similar to the one employed in the present study) or blocking the postsynaptic effect of activity in this pathway (by administering DA receptor blockers) results in symptoms similar to those of Parkinson's disease, such as akinesia, rigidity, tremor. These symptoms are improved by anticholinergic drugs, whereas acetylcholinesterase inhibitors exacerbate them inboth man and animals (179). Thus, evidence for the postulated functional balance between antagonistic striatal DAergic and cholinergic systems continues to grow. The exact nature and the anatomical arrangement of the systems involved is still unclear. Such

a functional balance implies antagonistic DAergic and cholinergic inputs on certain striatal cells or an interconnection between striatal DAergic and cholinergic neurons, as suggested by experiments mentioned above and by the present experiments (as illustratated in the diagram of Fig. 2.2). An imbalance of these systems towards a cholinergic preponderance, because of, for example, impaired DAergic function (following lesions of the substantia nigra or administration of DA receptor blockers) or enhanced cholinergic activity (secondary to administration of cholinomimetic drugs), leads to parkinsonian symptoms.

Acute treatment of rats with apomorphine or haloperidol did not change the striatal cholinergic imbalance induced by the lesion in the present study (Table 2.3). The CAT activity of the denervated striatum was significantly higher than the activity of the intact side following the administration of either of the two drugs. Similarly Guyenet et al. (93) following the same experimental procedure, found that these drugs affected the concentration of Ach on the intact and the denervated side of the striatum to the same extent.

The postsynaptic DA receptors, located on cholinergic or other interneurons of the striatum (94) did not seem to give a 'supersensitive' response to DA receptor stimulants. Therefore, the biochemical events, such as the activity of the cholinergic neurons, which are probably influenced by the DAergic neurons through these receptors, do not correlate with the phenomenon of 'supersensitivity'. The effect of denervation on CAT was probably due to the disinhibition of the cholinergic neurons, following the destruction of the vast majority of the DAergic fibres. Increased number of postsynaptic DA receptors (119) or increased activity of DA-sensitive adenylate cyclase (116), probably associated

with DA receptors or hyperactivity of the remaining intact DAergic neurons (123) do not seem to be responsible for the increase in CAT, since the DA receptor blocker haloperidol seemed to produce in the intact side a similar increase but had no effect in the denervated side (Fig. 2.1)

The suggestion - that the changes in Ach turnover and levels and in CAT activity induced by administration of DA receptor agonists and antagonists are due to the ability of these drugs to alter DAergic influence and are not mediated by a direct effect on the cholinergic system - gains support from the observation that these drugs, although producing marked effects in the striatum, have no effect on Ach concentration or turnover in areas not innervated by the DAergic system (184). These and the present experiments thus provide strong evidence for the concept that cholinergic neurons in the striatum are under the influence of a tonic inhibitory input from the DAergic neurons which have their cells of origin in the pars compacta of the substantia nigra.

Additional evidence comes from the work of Marco et al.(184), who found that haloperidol increased the Ach turnover rate in the rat striatum. This further supports the current belief that nigrostriatal DAergic synapses inhibit the metabolism of Ach in cholinergic postsynaptic interneurons. When the DAergic receptors are blocked by a neuroleptic, the metabolism of Ach in the striatum is increased, suggesting an increase of the activity of the intrinsic cholinergic interneurons. The increased CAT in the intact striatum after haloperidol probably involves an increased activity of existing CAT, rather than induction of new enzyme, because of the change in response to the treatment. On the contrary, the lesion-induced increase is more likely to reflect induction of new enzyme and increased concentration in the denervated striatum.

The discrepancy found by Guyenet et al. (93), when surgical and pharmacological interruption of the nigrostriatal DAergic pathway were compared does not rule out an inhibitory influence of DAergic neurons on the cholinergic system. Their results suggest, however, that the physiological release of DA exerts a modulation of the activity of cholinergic neurons much less pronounced than the effects induced by large doses of a neuroleptic drug or apomorphine. The slight but not significant decrease of Ach concentration observed by these investigators after degeneration of the nigrostriatal DAergic pathway contrasts with the marked reduction found by other workers (181) and does not explain the results of the present study. The completeness of the lesion, the time elapsed after the lesion and other methodological points could be the reason for this disagreement of the data. Failure to detect changes in the CAT activity of the striatum after various treatments, however, may be due to the fact that the levels of the enzyme in the striatum are very high, and a small but definite enzyme change due to severing the nigrostriatal projection might go undetected.

Other complementary approaches, such as autoradiographic or immunohistochemical studies, have to be used to establish the occurrence of direct interactions of cholinergic neurons with the DAergic terminals in the striatum and their possible importance as local regulatory processes. If, in addition to the cholinergic interneurons whose activity seems to be controlled (partly at least) by the nigrostriatal DAergic pathway at a postsynaptic level, other cholinergic neurons impinge at a preterminal level on this pathway, a more complex system might evolve. Indeed, Ach and other cholinergic agonists induce important changes in the release of DA from DAergic terminals, as has been shown in striatal

slices in vitro (174). The striatum is devoid of DAergic cell bodies. Therefore, it can be assumed that Ach and cholinergic agonists exert their effects on DAergic terminals by acting directly on these terminals or through local interneuronal processes. The changes in DA release induced by these drugs could be prevented by nicotinic or muscarinic blockers at low concentrations, indicating that these effects were mediated by nicotinic and/or muscarinic receptors. Therefore, it could be postulated that there are nicotinic and muscarinic presynaptic receptors on DAergic terminals in the striatum. Interactions mediated through these receptors could play an important physiological role, contributing to local regulatory processes. The activation of intrastriatal cholinergic neurons induced by the blockade of DAergic transmission (following lesions or neuroleptic treatment) may be hypothesised to trigger a stimulation of DA release, finally resulting in a reduction of the cholinergic neuronal activity. The impairment of this mechanism by an almost complete lesion of the nigrostriatal pathway may remove this process (which seems to control the cholinergic activity in the striatum) and lead to the observed increase in CAT, the marker of activity of cholinergic neurons.

Several questions remain unanswered, however, with regard to the sites and the mechanism of these interactions. The lack of evidence concerning the number of cholinergic receptors in the denervated striatum, the completeness of the lesion, the possible change in the sensitivity of striatal cholinergic receptors induced by the lesion, and the separation of cholinergic receptors located at pre- or postsynaptic level, does not allow the elucidation of the exact mechanism of DA-Ach interaction in the striatum. Further experiments are needed in order to clarify these points.

2.4.4 Dopamine-GABA interrelation in the striatum

A similar pattern of changes in GAD enzyme activity was found in the striatum after 6-OH-DA lesions of the nigrostriatal DAergic pathway. Thus, the activity of the enzyme was signigicantly elevated in the denervated, compared to the intact side of the striatum. A high dose of apomorphine (2 mg/kg i.p.) had no significant effect on either side of the striatum, but the GAD activity became not significantly different between the two sides after this treatment. A relatively high dose of haloperidol (1 mg/kg i.p.) produced a significant decrease of the enzyme activity only in the intact side, thus increasing the lesion-induced imbalance.

Although the mechanism of the enzyme protein induction may be the same for both CAT and GAD, the effect of the lesion and the various drug treatments on their activities is not the same. This is not surprising, in view of the known different neuronal links between the DAergic and the cholinergic or the GABAergic system in the striatum. In contrast to the well established direct synaptic link between the nigrostriatal DAergic nerve terminals and the striatal cholinergic interneurons, there is no anatomical or other evidence for a direct contact of the DAergic pathway with the striatal GABA-containing cells which give rise to the descending striatonigral or striatopallidal pathways. If the suggestion that DAergic terminals do not have functionally important direct links with GABAergic neurons in the striatum (106) is accepted, then the GAD changes after lesions of the substantia nigra might be a consequence of a disturbance of the cholinergic system. This would suggest the interesting possibility of a DA-Ach-GABA chain in the striatum (Fig. 2.2).

Recent pharmacological evidence, however, suggests that DAergic

neurons may have a 'trophic' influence on the GABA-containing neurons in the striatum. DA receptor agonists and antagonists have been shown to increase or decrease, respectively, the activity of striatal GABAergic neurons (105,129,184). Whether this stimulatory effect is direct or indirect (mediated through cholinergic interneurons) is still a matter of speculation. The possible neuronal links are depicted in Fig. 2.2.

Other evidence, in addition, suggests that GABA may have a regulatory influence on the DAergic pathway, either in the striatum (at a preterminal level) or in the substantia nigra. Thus, unilateral intranigral injection of picrotoxin, a presumed GABA receptor blocker, produced a reversible contralateral turning syndrome (191), similar to that seen after nigrostriatal pathway lesions. Furthermore, intranigral GABA administration caused a reduction of DA turnover in the striatum (130), whereas bicuculline, a GABA receptor blocker, enhanced the release of striatal DA (192). The presented evidence for an effect of GABA on DA release in the striatum (192,194) adds further support to the wealth of data suggesting a DA-GABA interaction in the striatum and the substantia nigra.

The sites of interaction may be: the substantia nigra, where descending GABAergic striatonigral and pallidonigral fibres terminate; the striatum, where the DAergic fibres may have postjunctional contacts with GABAergic neurons (either directly, or indirectly through cholinergic interneurons) or prejunctional contacts with GABAergic neurons impinging on them. With regard to the proposed concept of presynaptic control of DA release by GABA, the available evidence probably favours the inhibitory effect of the GABA neurons (192), although a stimulatory effect has also been suggested (194). Through their intrastriatal terminals, GABA neurons (probably axon collaterals of the striatonigral pathway) could influence directly or indirectly DA release from

DAergic terminals. GABA could act as an excitatory or an inhibitory transmitter and would induce a depolarisation or a hyperpolarisation, depending on the intracellular chloride concentration (195). These, and other possible sites and mechanisms of interaction of DA and GABA have to be borne in mind, in attempting to interpret the results of lesions of the nigrostriatal pathway on the GABA system.

The results of the present study indicate that GABA- or GAD-containing neurons of the striatum are not damaged by the lesion and subsequent degeneration of the nigrostriatal DAergic pathway. On the contrary, the demonstrated increase in GAD activity and in GABA concentration (if one can rely on this parameter, despite the reservations mentioned in the Introduction, 2.1.8) support an inhibitory influence of DAergic neurons on the striatal GABA activity. Whether this influence is direct or indirect cannot be decided from the present experiments, but there is evidence against a direct synaptic contact at the striatal level (106). Permanent interruption of the nigrostriatal pathway and elimination of the DAergic neuronal transmission seemed to disinhibit the GABA neurons of the striatum and cause increased synthesis and accumulation of GABA. Apomorphine had no effect on the GAD activity or the GABA concentration in the denervated side of the striatum, whereas it appeared to increase significantly the GABA concentration in the intact side. This finding gives further support to the belief that presynaptic DA receptors mediate the biochemical effects of apomorphine and is in agreement with the lack of effect of this agent on DA metabolism in the denervated striatum (Table 2.2). Haloperidol, which is believed to act mainly by inhibiting the postsynaptic DA receptors did not have a significant effect on GAD activity or GABA concentration on the denervated side,

although it decreased significantly these parameters on the intact side.

2.4.5 Dopamine-acetylcholine-GABA link (Fig 2.2)

Thus, it seems that the activation or blockade of postsynaptic DA receptors in the striatum leads to 'supersensitive' behavioural responses, which are not accompanied by any 'supersensitive' biochemical responses. These biochemical effects, which involve the DAergic, the cholinergic and the GABAergic neuronal systems would be rather mediated through stimulation of the presynaptic DA receptors, as their elimination after degeneration of the presynaptic sites indicates. Alternatively, the prejunctional (presynaptic) links of cholinergic or GABAergic neurons with the DAergic pathway at the striatal level may exist in addition to the postjunctional (postsynaptic) contacts of the DAergic nerve terminals with the other two systems. The demonstrated release of DA by Ach in the striatum (174) and the stimulation (194) or inhibition (192) of release of DA by GABA, suggested by other investigators, point to the existence of prejunctional links, probably capable of modulating the function of the DAergic terminals in the striatum. Furthermore, unilateral 6-OH-DA lesion of the substantia nigra resulted in a significant reduction of the GABA receptor binding in the ipsilateral striatum (196), suggesting that some of the GABA receptor binding sites are located on DAergic axons. On the other hand, the finding that adenylate cyclase, thought to be associated with the postsynaptic DA receptor, is located on intrinsic striatal neurons and destruction of this enzyme with kainic acid leads to significant decrease of 70% of the neurochemical markers for the cholinergic (CAT, Ach, Ach uptake) and GABAergic (GAD, GABA, GABA uptake) neurons in the striatum (94)

suggests that there are direct postsynaptic contacts of DAergic nerve terminals with both GABAergic and cholinergic neurons in the striatum.

On the basis of the available evidence, it could be postulated that DAergic terminals in the striatum form synapses with intrinsic cholinergic and GABAergic neurons and that these neurons in turn form synapses with the DAergic axons at a preterminal level, thus regulating the synthesis and release of DA. Degeneration of the DAergic pathway disinhibits the two systems, causing an increase in their neuronal activity, as implied by the increased CAT and GAD activities in the denervated striatum. Therefore, a local feedback control system seems to be indirectly activated to modulate the activity of the remaining intact DAergic nerve terminals. However, this control system might be inactivated as a result of the destruction of the vast majority of the DAergic fibres and the degeneration of the Ach or GABA presynaptic binding sites, or it might be maximally activated as a result of DA receptor supersensitivity. This could be particularly true for a cholinergic presynaptic control of DA release. The activated after the lesion cholinergic neurons would increase the DA release from the intact DAergic nerve terminals, thus leading to the observed marked increase of DA turnover and the disproportional reduction in the concentrations of DA metabolites and DA in the lesioned striatum. The demonstrated reduction of GABA receptor binding sites in the striatum after 6-OH-DA lesions of the substantia nigra (196) also seems to support this scheme. Assessment of any changes in the number of other properties of cholinergic receptors in the striatum after 6-OH-DA lesions of the substantia nigra could clarify further the extent of cholinergic participation in this postulated local feedback system.

2.4.6 The functional interactions in the substantia nigra (Fig. 2.2)

However, an integrated picture of the neuronal interconnections could be obtained only if the substantia nigra was included in the biochemical studies and the findings examined in the light of the known anatomical connections of the substantia nigra and the striatum.

The complexity of the nigral processes involved in interactions with the DAergic neurons is obvious from the numerous reports based on anatomical, physiological, biochemical, pharmacological and lesion studies. The present study confirmed the interdependence of the DAergic, GABAergic and cholinergic mechanisms in the substantia nigra. Lesion of the left side of this region with 6-OH-DA caused a significant fall in CAT, GAD and GABA compared to the right side (Table 2.4, Fig. 2.1). Administration of the DA receptor blocker haloperidol did not alter the CAT activity of any side, but it increased significantly the GABA concentration and the GAD activity of the lesioned side (compared to the same side in saline treated animals) whereas it had no significant effect on GABA and GAD in the intact side of the substantia nigra. Thus, haloperidol appeared to stimulate preferentially the activity of the GABAergic neurons in the lesioned side of the substantia nigra, and to reverse the lesion-induced imbalance of the two sides.

Apomorphine reduced equally the CAT activities in both sides and, therefore, did not alter the cholinergic imbalance induced by the lesion. A disagreement between the GAD and GABA changes was noted after administration of apomorphine: The GAD activity was increased in the intact side but not in the lesioned side (compared to the same sides in saline treated animals), resulting in an even bigger difference between the two sides; the GABA concentration, however, was not changed by this

treatment in the intact side, but was significantly elevated in the lesioned side, resulting in an imbalance opposite to that seen in GAD i.e. the GABA concentration of the left became significantly higher than that of the right side. Thus, apomorphine appeared to reverse the lesion-induced imbalance of the two sides of the substantia nigra in relation to GABA, but to increase the imbalance even further in relation to GAD.

The evidence from the effects of apomorphine and haloperidol on GABAergic activity in the substantia nigra offers a close biochemical correlate of the 'supersensitivity' phenomenon. The finding of decreased GAD and accumulated GABA in the lesioned side (compared to the intact side) after apomorphine, i.e. the reverse of the lesion-induced changes, and the contralateral turning of the rats, induced by the same drug, are reminiscent of the contralateral turning induced by unilateral intranigral injection of the GABA receptor antagonist picrotoxin (191). The reversal of the effect of apomorphine on nigral GAD by haloperidol (which can induce ipsilateral turning of the lesioned rats), also supports the same contention, i.e. that apomorphine acts as a GABAmimetic and haloperidol as an anti-GABAmimetic agent in the substantia nigra.

The assumption could be made that an increase in GABA concentration not followed by a change in GAD activity (or GABA transaminase activity) might imply an increase in GABA release, whereas a parallel increase or decrease of GABA and GAD might suggest an effect on the turnover of GABA. Similarly, an increase in GAD activity not accompanied by a change in GABA concentration might mean an increase of turnover. Parallel changes in the activities of the synthesising and the degradative enzymes may not have any effect on GABA concentration.

It has been demonstrated that intranigral 6-OH-DA injections may have non-specific toxic effects on other neurons at the injection site, in addition to the effect on the DAergic neurons (198,199). A non-specific effect could, therefore, contribute to the observed in the present study reduction of cholinergic and GABAergic activity in the lesioned side of the substantia nigra. However, the finding that the DA receptor agonist apomorphine and the DA receptor blocker haloperidol can influence in a predicted manner the markers of cholinergic and GABAergic neurons in the lesioned side indicates that, at least some of these changes, result indirectly through the primarily lesioned DAergic neurons. The finding that 6-OH-DA lesions of the substantia nigra did not affect the activity of the DA-sensitive adenylate cyclase, thought to be located on GABA- or substance P- containing neurons (56, 46) also supports the proposition that the effects on the GABAergic and cholinergic neurons of the substantia nigra may result indirectly, following a primary destruction of the DAergic neurons.

The already proposed model of DA-GABA interrelation in the substantia nigra (Section 1) may be again recalled and examined in the light of the findings described in this Section. The evidence for the existence of close synaptic contacts between GABAergic terminals and DAergic dendrites (32), the strong evidence for the inhibitory effect of GABA on nigral DAergic neurons (64) and the demonstrated GABA release by DA in vitro from slices of rat substantia nigra (57) suggest that DA released from the dendrites may interact with DA receptors located on terminals containing GABA, and the consequent selective release of GABA may cause the observed inhibition of DAergic neurons. The inhibitory effects of DA on DAergic cells when applied microiontophoretically into the substantia nigra (67) may, at least in part, be mediated by this releasing action of DA on GABA-containing nerve terminals.

Furthermore, the occurrence of GABA receptors on DA cell bodies or dendrites was suggested by the loss of ^{3}H-GABA binding specifically in the substantia nigra of Parkinsonian patients (202). This might be the postulated site of inhibitory action of the released GABA on the DA-containing neurons.

The concept of an inhibitory GABAergic input on DAergic cell bodies, which regulates the activity of the nigrostriatal DAergic pathway (44) has been strongly disputed as a result of the finding that lesions of the striatonigral GABAergic pathway do not affect the striatal DA turnover, either in control or in haloperidol or apomorphine treated animals (165, 164). The evidence that post-synaptic DA receptors are not a prerequisite for the effects of DA receptor stimulants and blockers on striatal DA metabolism (166) casts further doubt on the idea of a neuronal striatonigral feedback pathway regulating the activity of the nigrostriatal DAergic pathway. The proposed local autoregulatory mechanisms (203) seem to participate in the control of the activity of DA-containing neurons in the striatum and the substantia nigra.

Degeneration of the vast majority of the DAergic neurons in the substantia nigra, e.g. by 6-OH-DA injection, would be expected, according to the proposed model, to lead to inactivity of the GABAergic nerve terminals which have close synaptic contacts with them. This might explain the decrease in both markers of GABAergic neurons in the lesioned substantia nigra (i.e. GAD activity and GABA levels) found in the present study, which indicates a decreased GABA activity. Systematic administration of apomorphine produced, probably, an increase of the GABA turnover in the intact side of the substantia nigra (increased GAD activity, no significant effect on GABA concentration), further

supporting the stimulating effect of DA and DA receptor agonists on GABAergic neuronal activity in this structure (i.e. GABA mimetic action). On the lesioned side, however, there was an acutely-induced increase in GABA concentration but no effect on GAD, suggesting a 'super-sensitive' response of the DA receptors thought to be located on GABA terminals resulting probably in increased release of GABA (assuming a loss of GABA-transaminase due to the lesion). Thus, the 'super-sensitivity' phenomenon may not be only a mechanism which involves the DA receptors of the denervated striatum, but also the DA receptors of the lesioned substantia nigra. Further studies are needed in order to support this hypothesis and elucidate the mechanism of 'supersensitivity'. Various other parameters, such as DA metabolism, adenylate cyclase activity and GABA release from the substantia nigra lesioned with 6-OH-DA and following DA receptor stimulation, may be necessary for the understanding of the proposed nigral DA receptor 'supersensitivity'.

Haloperidol, given systemically, had no effect on GAD or GABA in the intact side, but it produced an increase in GAD activity and GABA concentration in the lesioned side. The latter suggests an increase in synthesis and accumulation of GABA as a consequence of DA receptor blockade in the lesioned substantia nigra. The paradoxical similarity of the effects of DA receptor stimulation and of DA receptor blockade on GABA levels in the lesioned substantia nigra cannot be readily explained, although it emphasises the complexity of the mechanisms involved. Haloperidol, by blocking the nigral DA receptors, probably located on GABA terminals (as the increase in nigral DA turnover indicates, Section 1; also, ref. 54) might be inducing a secondary activation of GABA synthesis, resulting in accumulation of GABA but not release (due to the lack of DA receptor stimulation).

The proposed model does not, however, take into account the possible involvement of substance P, (as speculated in Section 1), probably released by DA from the terminals of its striatonigral projections (46). Since substance P has been proposed as an excitatory neurotransmitter of the striatonigral projection (58), and other neurotransmitters such as Ach and 5-HT are believed to function in the substantia nigra, more work to study the integration of the various neurotransmitter systems is necessary for the understanding of the proposed local regulation of DA neuronal activity.

Since dendro-dendritic connections have been reported in the substantia nigra (73) and since some of these synapses may involve DAergic dendrites, it is possible that a cholinergic link may operate at some synapses (172). An initial, probably electrotonically induced, release of Ach would facilitate in turn the release of DA from presynaptic sites, and the released DA would act postsynaptically as a neurotransmitter, probably to release GABA or 5-HT from their neurons. The acetylcholinesterase found within or in the vicinity of dendrites of nigral DAergic neurons (205) might serve the important function of inactivating the initially released Ach. A similar function may be served by acetylcholinesterase found within or in the vicinity of the DAergic cell bodies in the pars compacta (205), i.e. to inactivate a cholinergic input. This scheme of Ach-DA functional link in the substantia nigra is supported by the finding of the present study that CAT activity is significantly lowered in the lesioned side of this structure, presumably as a result of inactivity of cholinergic neurons due to the degeneration of the target DAergic neurons. Observations that CAT in the substantia nigra (pars compacta) was significantly lowered in parkinsonian patients with characteristic degeneration of

nigral DAergic cells (206) also support this hypothesis.

Thus, the 6-OH-DA lesion of the substantia nigra had different biochemical effects on neuronal systems other than the DAergic system, in both substantia nigra and striatum. The difference could be explained by the different anatomical location and the functional interrelation of these systems. The idea of local presynaptic regulatory interrelations of the DAergic, cholinergic and GABAergic systems in the two areas seemsto explain various findings of this and other studies and avoids the controversial neuronal feedback loop hypothesis or the drawbacks of the exclusiveness of the 'DA autoreceptor' theory for the regulation of DA neurotransmission.

The finding that changes in GABA concentration and GAD activity in the substantia nigra, both in control and in apomorphine and haloperidol treated lesioned animals, do not correlate with changes in DA metabolism in the striatum, may be another piece of evidence against the neuronal feedback loop hypothesis which is based on the influence of the inhibitory, descending GABAergic striatonigral pathway on the ascending nigrostriatal DAergic pathway.

Finally, the possibility exists that glial cells modulate neuronal excitability via release of GABA (207, 208), through their contacts with nerve terminals and dendrites containing other neurotransmitters. The fact that there is almost no GAD present in the glial fractions of striatal homogenates (209) and the close correlation between GAD and GABA found in the present study suggest that mainly neuronal GABA is participating in the interactions with other neuronal systems. However, glial cells do seem also to have a DA-sensitive adenylate cyclase, probably linked with GABA release (209), indicating that glial GABA might also be involved in the regulation of neuronal activity.

2.4.7 Conclusions

1. Unilateral 6-hydroxydopamine lesions of the rat substantia nigra were effective in reducing by over 90% the concentration of DA in the corpus striatum, indicating a proportional degeneration of nerve terminals belonging to the nigrostriatal pathway.

2. Reduction of striatal DA by 90% was accompanied by reduction of striatal DA metabolite concentrations to only 70%; more complete lesions also resulted in non-proportional, higher reduction of HVA and DOPAC. These data may be indicative of an increased activity of the remaining intact neurons, which results in a marked increase of striatal DA turnover.

3. Administration of apomorphine had no effect on HVA or DOPAC in the partially (approx. 90%) denervated striatum, whereas it reduced both metabolites in the intact striatum. Haloperidol was capable of increasing the metabolite concentrations of both sides, with only a small deficit in the lesioned side.

The suggestion that apomorphine stimulates predominantly the presynaptic and haloperidol blocks the postsynaptic DA receptors in the striatum seems to be supported by these results. It appears that there is no supersensitivity of the presynaptic DA receptors located on the intact nerve terminals. The effects of haloperidol seem to suggest that the increased number and the increased responsiveness of the postsynaptic DA receptors may increase the activity of a local feedback system, probably resulting in an increase of DA release, even in the lesioned striatum. This feedback system may be cholinergic or GABAergic or utilising some other neurotransmitter.

4. Increased activity of the enzymes GAD and CAT, the neurochemical markers for the GABAergic and cholinergic neurons, respectively,

was found in the denervated (by approx. 90%) striatum, indicating a normally inhibitory influence of the nigrostriatal pathway on these neurons. No correlation was observed between the behavioural 'supersensitivity' phenomenon that follows the administration of apomorphine, and any postsynaptic biochemical changes. The data indicate a hyperactivity of the cholinergic and GABAergic neurons in the lesioned striatum, and support the concept of presynaptic modulation of DA neuronal activity by collaterals of cholinergic or GABAergic neurons impinging at a preterminal level on the nigro-striatal pathway.

5. The enzyme activities of GAD and CAT in the substantia nigra were reduced, indicating a possible dependence of the respective neurons on DA innervation. The data are consistent with a DA-GABA interrelation as proposed in Section 1 and may suggest a DA-Ach interaction at the nigral level.

6. The demonstrated reversal of GABAergic activity in the lesioned substantia nigra following the administration of apomorphine correlates with the behavioural supersensitivity phenomenon and the contralateral turning of rats with a 6-hydroxydopamine lesion.

7. The DA receptor agonist apomorphine appeared to have a GABAmimetic action in the substantia nigra, whereas the DA receptor antagonist haloperidol seemed to act as a GABA antagonist. These results indicate that DA may also be a GABA stimulant, probably acting by stimulation of DA receptors located on GABAergic neurons (terminals of striato- or pallido-nigral projections) to release GABA which in turn may bind to its specific receptors on DA cell bodies or dendrites to inhibit the cell firing rate and consequently the DA nueronal activity in the substantia nigra.

This model avoids the complexities of the 'striatonigral feedback loop' hypothesis for the regulation of DAergic activity and its ineffectiveness to explain certain experimental data. The 'DA autoreceptor' theory is also in doubt, as a result, since it does not sufficiently answer the questions concerning the association of adenylate cyclase and DA receptors in the substantia nigra and the interrelation of DA with other neurotransmitters.

SECTION 3

SOME BIOCHEMICAL EFFECTS OF CHRONIC TREATMENT OF RATS WITH NEUROLEPTICS ON DOPAMINERGIC, CHOLINERGIC AND GABA-ERGIC NEURONS OF THE CORPUS STRIATUM AND THE SUBSTANTIA NIGRA

3.1 INTRODUCTION

3.1.1 Therapeutic and side effects of neuroleptics

The short- and long-term effects of neuroleptic drugs differ both clinically and biochemically. In 1967 Schelkunov reported that, following chronic administration of a variety of classical neuroleptics (drugs which in animals induce catalepsy, antagonise apomorphine-induced stereotypy, and accelerate striatal DA turnover)(148) mice and rats showed increased and prolonged responses to amphetamine and apomorphine (70). The increased sensitivity to DAergic receptor stimulation persisted for several weeks after withdrawal of the neuroleptic agent. These findings have since been confirmed by other workers (72,78,80) and have been attributed to "chemical denervation" supersensitivity of the striatal DA receptors (74). Further it has been suggested that this mechanism could be responsible for the persistent and irreversible tardive dyskinesias appearing after prolonged administration of classical neuroleptic drugs (for several weeks or months but usually after several years of treatment) on reduction of dosage or on cessation of treatment (128).

There are two types of theories explaining the effect of neuroleptic drugs on DA metabolism. The receptor blockade hypothesis states that the neuroleptic drugs specifically attach to post-synaptic DA receptors in the nervous system (79) thus inhibiting activation of DA-sensitive adenylate cyclase (168), increasing the firing rate of DA neurons (67) accelerating DA

turnover (44) and blocking the effects of amphetamine and apomorphine (108). The coupling-blockade hypothesis states that neuroleptics act on presynaptic DA receptors to block the impulse-coupled release of DA thereby activating a feedback mechanism which increases DA turnover (163).

The neuroleptic drugs are widely used as antipsychotic agents. One characteristic of these drugs is their therapeutic latency. The drugs must be administered to patients for at least 2-3 weeks before the specific antipsychotic effects are seen; on the other hand, tardive dyskinesia and other side effects appear on chronic treatment with neuroleptic drugs (128). Many neurochemical changes are evident with short-term treatment with such drugs, particularly in DAergic systems in the brain, but the changes occuring with long-term treatment are probably less relevant to the therapeutic effects of these drugs. The neurochemical effects of long-term treatment with neuroleptic drugs have not been studied extensively. Those studies that have been made reveal striking differences between the biochemical effects of long-term and short-term drug treatment (e.g. 148,71).

Various attempts have been made to correlate the biochemical effects of chronic neuroleptic treatment with the clinical findings of antipsychotic effects and tardive dyskinesia, a syndrome characterised by abnormal movements of facial muscles and extremities, which is a major complication of long-term treatment with neuroleptic drugs (128). Lowering the dose or terminating the treatment frequently worsens these symptoms, while increasing the dose may alleviate the symptoms. Since a major action of neuroleptics is blockade of DA receptors in the brain, tardive dyskinesia is thought

to be linked to the phenomenon of 'supersensitivity' of DA receptors which develops after prolonged blockade by chronic neuroleptic treatment. This would explain why a reduction of dose worsens symptoms, while a dose increase temporarily reverses motor abnormalities. Studies in animals suggest that the increased motor activity reflects 'supersensitivity' of DA receptors. Rats or mice treated chronically with neuroleptic drugs show an increased sensitivity to the motor stimulant effects of apomorphine, a direct DA receptor agonist, after the treatment is terminated (72,78,80). A similar motor 'supersensitivity' to DA receptor stimulation is apparent when DA neurotransmission is reduced by inhibiting synthesis of DA with α-methyl-p-tyrosine or depleting DA stores with reserpine (72) or lesioning the nigrostriatal DAergic pathway (8,110).

Other neurological syndromes, such as acute dystonias, akathisias and drug-induced Parkinsonism, have also been associated with the use of neuroleptic drugs. These syndromes, which develop early in treatment and tend to diminish with time (128) are known as extrapyramidal side effects (EPS). They seem to be more relevant to the antipsychotic effects, since both appear early in treatment contrary to tardive dyskinesia which develops usually after several years of treatment (128). Tolerance develops to these EPS but not to the antipsychotic action of haloperidol (83). Thus one might assume that any biochemical change elicited by haloperidol, which shows tolerance may be related to the EPS and changes which persist after prolonged administration of haloperidol may reflect synaptic mechanisms related to its therapeutic action.

Both the clinical motor abnormalities seen after prolonged administration of neuroleptics in man (tardive dyskinesia) and the

increased response to apomorphine seen after such treatment in rats suggest that DA receptor sites may be supersensitive. However, behavioural supersensitivity to DA could be produced by a variety of other mechanisms, such as effects on non-dopamine neuronal systems, metabolic changes in cells at sites post-synaptic to DA neurons, or behavioural conditioning phenomena.

Various attempts have been made to correlate biochemically the phenomenon of tolerance to EPS produced after prolonged administration of neuroleptics, to distinguish EPS and therapeutic effects with regard to their biochemical correlates, to localize these effects, to define the neuronal system (or systems) affected and finally to elucidate the mechanisms of the EPS and the therapeutic-antipsychotic effects.

3.1.2 Aim of the study

It is well established that one of the major actions of neuroleptics is the blockade of DA receptors in brain, and that the EPS are also probably related to the blockade of DA receptors in the striatum. Therefore the effects of acute and chronic treatment with the neuroleptic agents haloperidol and α-flupenthixol (168) on the activity of the nigrostriatal DAergic pathway were investigated. The effects of chronic treatment with these agents on the activity of the cholinergic and GABAergic systems in the striatum and in the substantia nigra were studied and compared to the effects of acute treatment in order to define the involvement of these systems in the EPS and the therapeutic effects. Conversely, the nature of the neuronal interrelations was examined and, particularly, the role of the cholinergic and GABAergic systems in the regulation of the activity of the

nigrostriatal pathway. Finally, a comparison of the 'supersensitivity' phenomena demonstrated after lesions of the nigrostriatal DAergic pathway and after prolonged neuroleptic treatment was attempted, aiming at defining the interconnection of the DAergic with the cholinergic and the GABAergic neuronal systems in the striatum and the substantia nigra. The possible rearrangement of the neuronal interrelations after prolonged treatment with neuroleptic agents was assessed.

3.2 MATERIALS AND METHODS

3.2.1 Drug treatments

Male albino wistar rats (150-250 g) were used. In the acute experiments they were injected i.p. with haloperidol (1 mg/kg), α-flupenthixol hydrochloride (1 mg/kg), apomorphine hydrochloride (1 mg/kg or 2 mg/kg), DL-amphetamine sulphate (5 mg/kg) or saline solution (0.9% NaCl). The animals were killed 30 or 60 min after haloperidol, 180 min after α-flupenthixol, 30 min after apomorphine and 30 min after DL-amphetamine administration.

For chronic treatments, groups of animals were injected with haloperidol or α-flupenthixol at the dosage of 1 mg/kg i.p. daily between 10 and 11am for 15 days. Appropriate controls were injected daily with saline solution. All the animals were housed in groups of 4 - 5 under controlled temperature and had access to food and water ad libitum. After the final (15th) injection, the animals were killed as follows: The haloperidol-treated groups at 1 hour, 1, 3 and 6 days after the last injection; the α-flupenthixol-treated groups at 3 hours after the last injection.

The rats were killed by decapitation, the brains rapidly removed and the corpus striatum and the substantia nigra were quickly dissected out (within about 3 min) as described in Section 1, immediately immersed into liquid nitrogen and stored frozen until the biochemical analyses.

Apomorphine hydrochloride was purchased in solid form from Macfarlane Smith Ltd; haloperidol in ampoules as a commercially

available solution ("Serenace") from Janssen Pharmaceutica; α-flupenthixol hydrochloride in solid form from Lundbeck Ltd; DL-amphetamine sulphate in solid form from Sigma Chem. Co.

3.2.2 Biochemical determinations

The concentrations of homovanillic acid (HVA), 3,4-dihydroxy-phenylacetic acid (DOPAC) and γ-aminobutyric acid (GABA) and the activities of the enzymes choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) were determined in pooled tissues from both sides of the substantia nigra and the corpus striatum, according to the methods described elsewhere in this Thesis (Appendix for HVA-DOPAC, Section 2 for GABA, CAT, GAD).

Protein content of brain tissues was estimated in duplicate 5 µl aliquots of homogenates, according to the method of Lowry et al. (144).

3.3 RESULTS

3.3.1 Striatal DA metabolism after acute and chronic neuroleptic treatment

Acute treatment of rats with haloperidol (1 mg/kg i.p.) 30 min before sacrifice produced a highly significant increase in the concentrations of HVA and DOPAC in the corpus striatum to 363 and 278 per cent, respectively, of the concentrations in the striatum of saline-treated controls (Table 3.1). Similarly, the neuroleptic agent α-flupenthixol, given at a dose of 1 mg/kg i.p. 3 hours before sacrifice, caused a significant rise in both HVA and DOPAC, the concentration being, respectively, 190 and 202 per cent of the concentrations in the saline treated animals ($p < 0.01$ and $p < 0.0025$, respectively) (Table 3.1).

The increase in the striatal concentrations of HVA and DOPAC seen after acute treatment tends to disappear after chronic treatment with these neuroleptics. Thus, when rats were treated chronically for 15 days with a daily i.p. dose of 1 mg/kg haloperidol or α-flupenthixol, the initial increase observed after acute treatment was reduced (Table 3.1). The striatal HVA concentrations at 30 min, 1, 3 and 6 days after the last (15th) injection of haloperidol were 157, 145, 89 and 76 per cent, respectively, of those of saline-treated controls. All these HVA concentrations were significantly lower than that of this metabolite at 30 min following a single administration of haloperidol at the same dose level ($p < 0.0025$). The DOPAC concentrations showed a similar pattern to the changes in the HVA

Table 3.1

Effect of acute and chronic administration of neuroleptic drugs on the concentration of HVA and DOPAC in the corpus striatum

Drug and dosage	Type of treatment	Time	HVA µg/g tissue	% of control	DOPAC µg/g tissue	% of control
saline	acute	30 min	1.33 ± 0.30(7)	100	1.26 ± 0.17(7)	100
haloperidol 1 mg/kg i.p.	acute	30 min	4.83 ± 0.60(12)††	363	3.50 ± 0.56(12)††	278
saline	chronic	30 min	1.26 ± 0.17(5)	100	1.34 ± 0.29(5)	100
haloperidol 1 mg/kg i.p.	chronic	30 min	1.98 ± 0.31(5)$^{**}_{a}$	157	2.01 ± 0.25(5)$^{**}_{a}$	150
		1 day	1.83 ± 0.17(5)†	145	1.91 ± 0.24(5)**	142
		3 days	1.12 ± 0.19(5)	89	1.43 ± 0.15(5)	107
		6 days	0.95 ± 0.09(5)	76	0.92 ± 0.08(5)**	69
saline	acute	3 hours	1.11 ± 0.33(4)	100	1.18 ± 0.26(4)	100
α- flupenthixol 1 mg/kg i.p.	acute	3 hours	2.11 ± 0.26(5)**	190	2.39 ± 0.30(5)†	202
saline	chronic	3 hours	1.07 ± 0.10(6)	100	1.13 ± 0.16(6)	100
α- flupenthixol 1 mg/kg i.p.	chronic	3 hours	1.49 ± 0.30(6)$^{*}_{a}$	139	1.39 ± 0.18(6)$_{b}$	123

Legend for Table 3.1

The rats were killed at the indicated time after an acute or after the last of 15 daily injections of the neuroleptic drugs.

Control animals were injected saline solution.

Values represent means ± s.d.; the number of animals in each group is in parentheses.

Significance of difference from saline-control values (Student's t test; two tailed):

* $p<0.05$; ** $p<0.025$; $\overset{**}{*}$ $p<0.01$; † $p<0.0025$; †† $p<0.0005$;

a $p<0.0025$ compared to acute haloperidol;
b $p<0.025$ compared to acute α-flupenthixol.

concentrations in both the acute and chronic treatments (Table 3.1). Similarly, the increase to 190% for HVA and 202% for DOPAC of the concentrations in the striata from saline controls produced by a single administration of α-flupenthixol, was reduced to 139 and 123 per cent respectively when the animals were decapitated 3 hours after the last of 15 daily injections of this neuroleptic. Again, there was significant difference between the extent of the changes in the concentrations of both metabolites ($p < 0.025$) under the two conditions of drug administration (Table 3.1).

3.3.2 Nigral DA metabolism after acute and chronic neuroleptic treatment

In the same experiments, in parallel with the investigation of striatal DA metabolism, HVA and DOPAC were also estimated in the substantia nigra. As described in Section 1, 30 min after administration of haloperidol (1 mg/kg i.p.) there was an initial significant decrease in the concentrations of HVA and DOPAC, which was then followed by an increase which persisted for about 2 hours before declining to normal levels within 5 hours after the injection (Fig. 1.3). This pattern of the earlier changes was confirmed in the present series of experiments. Haloperidol given acutely (1 mg/kg i.p.) produced within 30 min a decrease of both HVA and DOPAC to about two-thirds of the control concentrations. One hour after the injection, the concentrations of both HVA and DOPAC were significantly elevated to about 2 times the saline control levels (Table 3.2).

After chronic treatment with haloperidol (1 mg/kg i.p.) for 15 days, the HVA and DOPAC concentrations in the substantia nigra were significantly higher than those from controls similarly chronically

Table 3.2

Effect of acute and chronic administration of neuroleptic drugs on the concentration of HVA and DOPAC in the substantia nigra

Drug and dosage	Type of treatment	Time	HVA µg/g tissue	% of control	DOPAC µg/g tissue	% of control
saline	acute	30 min	1.55 ± 0.26(8)	100	1.18 ± 0.15(8)	100
haloperidol 1 mg/kg i.p.	acute	30 min	1.04 ± 0.30(12)**	67	0.80 ± 0.21(12) †	68
		60 min	3.54 ± 0.59(5)	228	2.48 ± 0.50(5)	210
saline	chronic	30 min	1.41 ± 0.20(5)	100	1.06 ± 0.25(5)	100
haloperidol 1 mg/kg i.p.	chronic	30 min	1.92 ± 0.21(5)**	136	1.86 ± 0.32(5)**	175
		1 day	2.18 ± 0.43(5)**	155	2.16 ± 0.35(5)†	201
		3 days	1.74 ± 0.41(5)	123	2.12 ± 0.29(5)†	200
		6 days	1.59 ± 0.38(5)	113	1.64 ± 0.38(5)**	155
saline	acute	3 hours	1.62 ± 0.26(5)	100	1.36 ± 0.34	100
∝- flupenthixol 1 mg/kg i.p.	acute	3 hours	1.70 ± 0.26(5)	105	1.42 ± 0.18(5)	104
saline	chronic	3 hours	1.41 ± 0.26(6)	100	1.06 ± 0.25(6)	100
∝- flupenthixol 1 mg/kg i.p.	chronic	3 hours	1.92 ± 0.33(6)*	136	1.56 ± 0.18(6)*	147

Legend for Table 3.2

The rats were killed at the indicated time after an acute or after the last of 15 daily injections of the neuroleptic drugs.

Control animals were injected saline solution.

Values represent means ± s.d.; the number of animals in each group is in parentheses.

Significance of difference from saline-control values (Student's t test; two tailed):

* $p<0.05$; ** $p<0.025$; *** $p<0.01$; † $p<0.0025$.

injected with saline (Table 3.2). Elevated levels were still apparent for at least 6 days after the last injection. The HVA concentration, compared to control, was 136%, 30 min after the last injection and 155% 24 hours later. By 3 and 6 days after the last injection, it had declined to 123 and 113 per cent, neither value being significantly different ($p < 0.05$) from the control. The DOPAC concentration was 175% of the control value 30 min after the last injection and rose somewhat higher to 200% by 24 hours, at which level it was maintained to at least the third day after the last injection. By three days later the concentration of DOPAC had declined to 155% but was still significantly higher than the control ($p < 0.025$).

Three hours after α-flupenthixol given acutely (1 mg/kg i.p.) there was no significant change in either the HVA or DOPAC concentrations. After chronic α-flupenthixol administration (1 mg/kg i.p.), in contrast to the lack of any acute effect, the concentration of HVA was 136% of control ($p < 0.05$) and that of DOPAC 147% of control ($p < 0.05$), 3 hours after the 15th injection (Table 3.2).

3.3.3 Effects of acute and chronic neuroleptic treatment on GABA concentration and glutamic acid decarboxylase (GAD) activity in corpus striatum and substantia nigra

Acute administration of haloperidol (1 mg/kg i.p.) 30 min before sacrifice of the rats did not produce any significant change in the concentration of GABA in the striatum (Table 3.3). Three hours after an acute injection of α-flupenthixol (1 mg/kg i.p.) there was a significant fall in GABA concentration in the same brain area ($p < 0.0025$). In the substantia nigra, neither of the two neuroleptics

Table 3.3

Acute and chronic effects of neuroleptics on GABA concentrations in the corpus striatum and the substantia nigra

Drug treatment	Type of treatment	Time	GABA (µg/g tissue)	
			corpus striatum	substantia nigra
saline	acute	30 min	120 ± 49(12)	415 ± 68(12)
haloperidol 1 mg/kg i.p.	acute	30 min	124 ± 17(6)	496 ± 101(9)
saline	chronic	30 min	105 ± 20(6)	391 ± 63(6)
haloperidol 1 mg/kg i.p.	chronic	30 min	61 ± 26 (6)*	552 ± 97(6)*
saline	acute	3 hours	108 ± 14(6)	375 ± 82(6)
∝-flupenthixol 1 mg/kg i.p.	acute	3 hours	54 ± 11(6)**	380 ± 62(6)
saline	chronic	3 hours	105 ± 20(6)	391 ± 63(6)
∝-flupenthixol 1 mg/kg i.p.	chronic	3 hours	81 ± 15 (6)	378 ± 43(6)

The rats were killed at the indicated time after an acute or after the last of 15 daily injections of the neuroleptic drugs.

Control animals were injected saline solution.

Values represent means ± s.d.; the number of animals in each group is in parentheses.

Significance of difference from saline-control values (Student's t test, two tailed):

* $p<0.025$; ** $p<0.0025$.

caused any significant change, given at the above dosage and time schedule (Table 3.3).

Chronic administration of haloperidol (1 mg/kg i.p. daily) for 15 days, produced a statistically significant fall in the striatal GABA concentration ($p < 0.025$, compared to control) and a significant rise in the concentration of GABA in the substantia nigra ($p < 0.025$, compared to control) when the rats were sacrificed 30 min after the 15th injection. α-Flupenthixol, given chronically at a dose of 1 mg/kg i.p. daily, did not produce any significant change in either the striatum or the substantia nigra, when the rats were sacrificed 3 hours after the last (15th) injection.

The activity of glutamic acid decarboxylase (GAD), the GABA-synthesising enzyme, was measured in the corpus striatum and the substantia nigra after acute and chronic treatment with haloperidol (Table 3.4). Acute administration of haloperidol (1 mg/kg i.p.) 1 hour before decapitation caused a significant reduction of GAD activity in the striatum ($p < 0.005$). Repeated administration of haloperidol at a dose of 1 mg/kg daily for 15 days, produced (in contrast to the acute administration) a significant increase in the striatal activity of GAD. The increase of activity was observed at 1 hour ($p < 0.05$) and at 24 hours ($p < 0.01$) after the last (15th) injection, and disappeared at 3 days. Acute administration of haloperidol (1 mg/kg i.p.) had no effect on GAD activity in the substantia nigra, but chronic administration for 15 days produced a significant increase when the rats were killed 1 hour, 1 day and 3 days after the 15th injection ($p < 0.005$, $p < 0.025$ and $p < 0.01$, respectively). By 6 days after the 15th injection the nigral GAD activity was normalised (Table 3.4).

Table 3.4

Effect of acute and chronic neuroleptic treatment on the activities of the enzymes choline acetyltransferase (CAT) and glutamic acid decarboxylase (GAD) in the corpus striatum and the substantia nigra

Drug treatment	Type of treatment	Time	Choline acetyltransferase (CAT)		Glutamic acid decarboxylase (GAD)	
			corpus striatum	substantia nigra	corpus striatum	substantia nigra
saline	acute	1 hour	17.52 ± 2.84(5)	6.13 ± 1.32(5)	39.95 ± 2.73(5)	71.35 ± 5.85(5)
haloperidol 1 mg/kg i.p.	acute	1 hour	24.04 ± 2.56(5)**	7.09 ± 0.64(5)	27.17 ± 5.20(7)†	69.53 ± 9.35(7)
5 mg/kg i.p.	acute	1 hour	21.40 ± 3.91(5)	4.99 ± 1.11(5)		
10 mg/kg i.p.	acute	1 hour	20.78 ± 3.16(5)	4.61 ± 1.61(5)		
saline	chronic	1 hour	18.80 ± 2.66(6)	6.58 ± 1.29(5)	38.04 ± 8.53(6)	72.83 ± 12.26(5)
haloperidol 1 mg/kg i.p.	chronic	1 hour	14.78 ± 2.45(6)*	4.26 ± 1.59(5)	49.31 ± 7.05(6)*	125.00 ± 17.56(5)†
		1 day	17.67 ± 1.17(6)	11.65 ± 2.70(5)**a	53.20 ± 3.61(6)**	99.45 ± 12.08(5)**
		3 days	17.75 ± 0.90(6)	8.00 ± 1.67(5)	38.23 ± 3.83(6)	99.70 ± 10.10(5)**
		6 days	15.73 ± 2.88(5)	6.70 ± 1.51(5)	37.70 ± 3.06(6)	75.40 ± 8.86(5)

The rats were killed at the indicated time after an acute or after the last of 15 daily injections of the haloperidol. Control animals were injected saline solution.

Results are expressed as nmoles Ach formed per mg protein per hour for CAT and as nmoles CO_2 liberated per mg protein per hour for GAD.

Values represent means ± s.d; the number of animals in each group is in parentheses.

Significance of difference from saline-control values (Student's t test, two tailed):
* $p<0.05$; ** $p<0.025$; ** $p<0.01$; $p<0.005$
a $p<0.005$ compared to mean value 1 hour after the last injection.

3.3.4 Effects of acute and chronic neuroleptic treatment on choline acetyltransferase (CAT) activity in corpus striatum and substantia nigra

Acute administration of haloperidol at doses of 1, 5 and 10 $mg/_{kg}$, 1 hour before sacrifice, produced changes in the enzyme activity which were not dose-dependent (Table 3.4). A single injection of 1 $mg/_{kg}$ i.p. caused a significant increase in the striatal CAT activity ($p < 0.025$) but it had no significant effect on the nigral CAT. Doses of 5 and 10 $mg/_{kg}$ i.p. produced lower but not significantly different than the control mean enzyme activities in the substantia nigra, and higher but not significantly different activities in the corpus striatum.

There was a significant reduction of CAT activity in the corpus striatum of rats killed 1 hour after the last of 15 daily injections of haloperidol (1 $mg/_{kg}$ i.p.) ($p < 0.05$). No difference from control activities was evident when estimated in animals at 24 hours - 6 days after the last injection.

In the substantia nigra enzyme activity appeared to be slightly lower than the control at 1 hour after the last injection; by 24 hours after the last injection the activity of CAT appeared to be higher than the control ($p < 0.01$). The difference in activities at these two times (i.e. 1 hour and 24 hours after the final injection) was significant ($p < 0.005$). The tendency towards a higher activity appeared to persist for at least 6 days after haloperidol treatment was terminated (Table 3.4).

3.3.5 Effects of acute apomorphine and amphetamine on GABA concentration and on GAD and CAT activities in corpus striatum and substantia nigra

Acute administration of apomorphine (1 mg/kg i.p.) 30 min before sacrifice produced a highly significant increase in GABA concentration in the corpus striatum ($p < 0.0005$) and a highly significant decrease in the substantia nigra ($p < 0.0005$). DL-amphetamine, on the contrary, at a dose of 5 mg/kg i.p. given 60 min before decapitation, produced a significant decrease of GABA in the corpus striatum ($p < 0.01$) and a significant increase in the substantia nigra ($p < 0.05$) (Table 3.5).

Acute apomorphine or DL-amphetamine administration at the same dosage had no significant effect on GAD activity in the corpus striatum, but both drugs produced significant increases in GAD in the substantia nigra ($p < 0.05$).

Apomorphine had no significant effect on striatal CAT activity whereas DL-amphetamine produced a significant reduction ($p < 0.025$). In the substantia nigra both drugs caused significant reductions of the enzyme activity ($p < 0.05$) (Table 3.5).

Table 3.5

Comparative acute effects of haloperidol, apomorphine and amphetamine on GABA concentration and on GAD and CAT activities in the corpus striatum and the substantia nigra

Treatment	Time	corpus striatum			substantia nigra		
		GABA	GAD	CAT	GABA	GAD	CAT
saline	30 min	142 ± 32(12)	46.05 ± 7.06(5)	17.78 ± 2.23(8)	415 ± 68(12)	78.95 ± 10.31(5)	6.67 ± 0.78(5)
haloperidol 1 mg/kg i.p.	60 min	147 ± 43(7)	30.17 ± 3.46(6)**	22.04 ± 3.66(5)**	496 ± 101(9)	71.84 ± 6.39(5)	7.79 ± 1.64(5)
apomorphine 1 mg/kg i.p.	30 min	219 ± 48(13)†	46.27 ± 6.99(7)	16.01 ± 1.10(7)	272 ± 81(13)†	93.83 ± 5.83(7)*	5.40 ± 1.11(7)*
amphetamine 5 mg/kg i.p.	60 min	92 ± 20(6)**	46.15 ± 8.66(5)	14.33 ± 1.88(5)**	500 ± 87(7)*	92.52 ± 7.59(5)*	5.50 ± 0.99(5)*

The rats were killed at the indicated time after injection of the drugs. Control animals were injected saline solution.

Results are expressed as: μg/g tissue for GABA; n moles CO_2/mg protein/hour for GAD; n moles Ach/mg protein/hour for CAT.

Values represent means ± s.d; the number of animals in each group is in parentheses.

Significance of difference fromsaline-control values (Student's t test, two tailed):

* $p<0.05$; ** $p<0.025$; ** $p<0.01$; † $p<0.0005$

3.4 DISCUSSION

3.4.1 Striatal DA metabolism after chronic treatment with neuroleptics

The present study confirmed that tolerance to the effects of neuroleptic agents (or antipsychotic agents) on DA turnover in the corpus striatum develops after prolonged administration. Both haloperidol and α-flupenthixol produced effects on HVA and DOPAC concentrations which were significantly smaller than the effects they produced after a single administration, although they were still higher than the controls immediately after the last injection; 24 hours later and up to 6 days after the last injection the HVA and DOPAC concentrations in the striatum became normal (Fig. 3.1). This phenomenon of tolerance has been demonstrated in cats and monkeys (126), in rabbits (71) and in rats (148) by several other investigators.

By acting on postsynaptic or presynaptic DA receptors, neuroleptics elicit marked changes in the synthesis and the release of DA in the nerve terminal area of the striatum. In great part, these effects are related to fluctuations in nerve impulse flow and are reflected in changes in the concentrations of the DA metabolites HVA and DOPAC. Therefore, estimation of these metabolites can be used as an index of the activity of the nigrostriatal DAergic pathway. Changes in their concentrations in the striatum after prolonged administration of DA receptor blockers could reflect changes in the reactivity of the pre- or post-synaptic DA receptors.

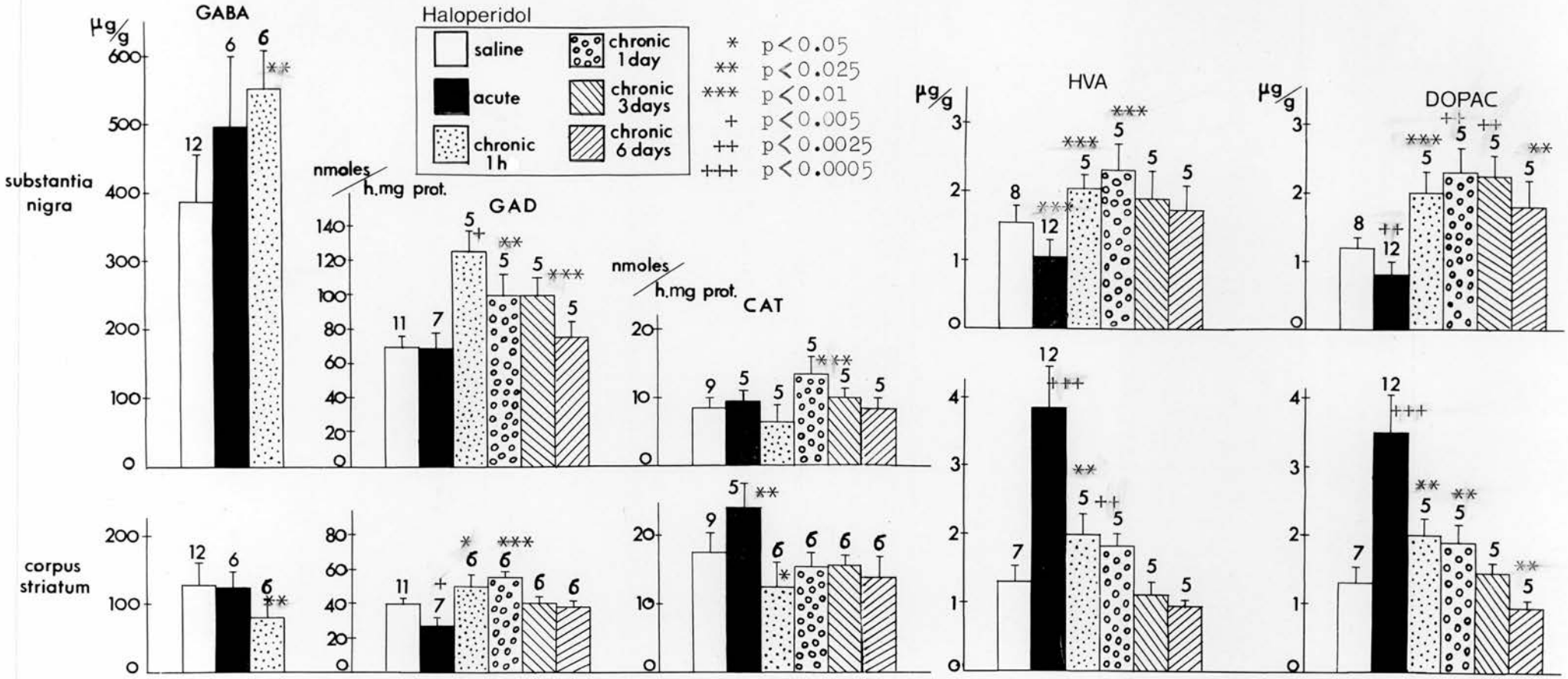


Fig. 3.1

Summary of biochemical effects of acute and chronic treatment of rats with haloperidol on the substantia nigra and the corpus striatum.

Rats were injected 1 mg/kg i.p. daily for 15 days and killed at 1 h , 1,3 or 6 days after the last injection.

Bars represent s.d. for the indicated number of animals.

Several workers have shown that a series of biochemical changes in the DAergic system occur after prolonged treatment with neuroleptics. The activity in the striatum of the enzyme tyrosine hydroxylase (the rate limiting step in DA biosynthesis) returned to baseline level 24 hours after the last in a series of daily injections of haloperidol, contrary to the significant increase seen after acute treatment (151). The synthesis of ^{3}H-DA from ^{3}H-tyrosine in striatal slices was markedly reduced 24 hours after the last in a series of daily injections of various neuroleptics (152).

In addition to these presynaptic effects of blockade of DA receptors in the striatum, an increase in the number of striatal DA receptors following chronic treatment of rats with neuroleptics (as evidenced by the increase in the number of ^{3}H-haloperidol binding sites) (153) suggests that postsynaptic changes may contribute to the observed tolerance. Thus the reactivity of the postsynaptic DA receptors in the striatum to acute or repeated administration of neuroleptics may be different. Dopaminergic neurons in this structure seem to adapt to repeated doses of neuroleptics, and this adaptation may represent a tolerance phenomenon which may actually be involved in the onset of the desired therapeutic effects or the disappearance of certain extrapyramidal side effects or both (78,83). The loss of sensitivity of striatal DA receptors to neuroleptics after prolonged administration is accompanied by the development of hypersensitivity to DA receptor stimulants, suggesting a functional modification of striatal DA receptors. However, the activity of a DA-sensitive adenylate cyclase in the striatum is not altered in rats treated chronically with neuroleptics (80). Moreover, the ability of apomorphine to increase striatal cyclic AMP concentrations in vivo is unaffected by the

treatment (80). Data indicating a differentiation of receptor binding sites and associated adenylate cyclase may explain this discrepancy (153).

It has been suggested that the inhibition of DA synthesis seen in striatal DAergic terminals after repeated injections of neuroleptics could be related to the presence of a second interneuronal regulatory process distinct from that involved in the activation of DA synthesis classically observed after acute treatment (152). Further experiments are needed in order to define whether this alternative mechanism is totally or partially associated with the DA receptor supersensitivity, or with the effects on non-DA neuronal systems, or with changes post-junctional to DA neurons in the striatum or even with behavioural conditioning phenomena. Our subsequent experiments attempted to clarify some of these points.

It has been shown repeatedly that a state of increased responsiveness to DA develops when the DAergic transmission has been impaired by denervation (8), by decreased transmitter release (157) or by receptor blockade (78). It has also been reported by several investigators that animals of different species become tolerant to sedative (154), adrenolytic (70) and cataleptic (148) effects of neuroleptics, in addition to the tolerance to the increase in DA turnover and HVA-DOPAC seen in the striatum (148, 126). Bowers and Romitis (71), however, have found development of tolerance to the drug-induced increase in HVA concentration only in the striatum and not in the limbic and hypothalmic areas after chronic treatment with neuroleptics. Similarly, Julou et al.(152) have demonstrated that the synthesis of DA is markedly enhanced in the mesolimbic and mesocortical (frontal and cingular cerebral cortex) DAergic systems

shortly after the termination of chronic treatment, but no effect could be seen at this time in the nigrostriatal DAergic terminals. This observation is quite distinct from that seen after acute treatment, when the maximal effect on DA synthesis was seen in the striatum. The disappearance of the EPS induced by neuroleptics after the initial period of treatment or the onset of the therapeutic-antipsychotic effects could probably be related to the regional differences with regard to DA metabolism. The EPS may thus involve an effect at the striatal level, since their disappearance correlates with a decreased responsiveness of striatal DA receptors to neuroleptics and an increased sensitivity to DA receptor stimulants (80, 72). The antipsychotic effects may be related to DA receptor blockade in the mesolimbic or mesocortical areas, as suggested by Julou et al.(152), since the neuroleptics exert their blocking effect on cortical or mesolimbic DAergic receptors for long periods of time.

Finally, the phenomenon of tolerance (with regard to DA metabolism) does not seem to result from any direct action of the neuroleptics on enzymesof the catecholamine metabolism (catechol-O-methyltransferase or monoamine oxidase) (148) or from induction of microsomal oxygenases metabolising the drugs (197), since a reduction of DA turnover is not observed in most brain areas.

3.4.2 Nigral DA metabolism after chronic treatment with neuroleptics

A single dose of a neuroleptic drug to rats causes an increase in the firing rate of nigrostriatal DAergic neurons (67) and an increase in striatal DA turnover (44). Repeated administration has convincingly been found to cause a tolerance to the effect on DA turnover, and this was confirmed by the present study. It was of interest, therefore, to investigate the effects of chronic neuroleptic treatment on DA turnover

in the substantia nigra. Earlier studies (reported in Section 1) had shown biphasic effects of haloperidol on HVA and DOPAC in the rat substantia nigra, i.e. an initial reduction followed by a sharp increase and finally a return to baseline levels. Similarly, high doses of haloperidol (5-10 mg/kg) produced no significant effect, whereas low doses (0.4-1 mg/kg) produced an increase in the levels of the DA metabolites in this brain region. These fluctuations were not observed in the corpus striatum with the various doses and at various times (Fig. 1.2 and 1.3). The present experiments confirmed the initial fall in HVA and DOPAC in the substantia nigra following a single injection of haloperidol. The finding that the other neuroleptic used, α-flupenthixol, did not cause any effect on the metabolite levels when it was injected 3 hours before decapitation of the animals, although it produced an effect in the striatum, similar (but lower) to haloperidol, indicates that the effects of haloperidol are time- and dose-dependent. Prolonged administration (for 15 days) of both drugs resulted in no tolerance to the increase in DA metabolism in the substantia nigra. The HVA and DOPAC concentrations were markedly enhanced shortly after the last injection of the drugs and persisted for at least 24 hours, and even for 6 days with regard to DOPAC after the last injection of haloperidol (Fig. 3.1).

Thus, a discrepancy arises from the comparison of the changes in the corpus striatum and the substantia nigra. The increase in DA synthesis or turnover in the DA nerve terminal area of corpus striatum is thought to result from an increase in the firing rate of the DA cell bodies in the substantia nigra (25). Despite the demonstrated dose- and time-dependent deviations from this rule, a parallel increase in DA turnover seems to occur in the two ends of the nigrostriatal pathway

after acute haloperidol administration. The results of chronic administration, however, deviate from the rule. Although further experiments are needed, such as electrophysiological and receptor studies on the substantia nigra, one could hypothesise that the persistent increase in DA cell activity and firing rate, implied by the elevated levels of HVA and DOPAC in this region, was not followed by an increase in DA turnover or synthesis in the nerve terminal area of the striatum. Several recent reports suggest a reciprocity between the effects of DA release at the two ends of the nigrostriatal pathway. Using a push-pull cannula technique, Cheramy et al.(52) have shown that substantia nigra and corpus striatum react in opposite directions to sensory stimulation and to local stimulation. From electrophysiological studies it has been suggested that DA release in the substantia nigra may inhibit the firing rate of DA cells (53). Thus an increase in DA release in the substantia nigra would be associated with a decrease in the corpus striatum. Biochemical studies in rats treated with haloperidol (Section 1) suggested that the reverse may also be true, that is: an increase in DA release in the corpus striatum is associated with a decrease in DA release in the substantia nigra. The suggestion that the diminished activity of the nigrostriatal pathway after chronic neuroleptic treatment results from an increased activity of the 'feedback' striatonigral loop which exerts its inhibitory influence on the nigral DA cells (189) does not seem to explain the findings of the present study, since such an effect would rather reduce DA turnover in the substantia nigra as well as DA turnover in the striatum.

The proposed local feedback regulation of DA metabolism, mediated by the nigral DA 'autoreceptors' or by the release of

inhibitory neuromodulators, such as GABA, may offer an alternative interpretation. The prolonged blockade of nigral DA receptors, which are probably associated with the DA-sensitive adenylate cyclase found in this region (16,18), seems to result in an increase of the DA cell firing rate and consequently in an increase of synthesis and release of DA from the dendritic processes in the pars reticulata. The fact that the changes induced by the repeated neuroleptic treatment are not higher than the maximal effect induced by a single injection of haloperidol, may indicate that some kind of homeostatic mechanism exists in the substantia nigra, which adapts the sensitivity of the target DA cells to the inhibitory or excitatory inputs. Such a compensatory mechanism could be a supersensitivity of the DA-containing cells in the substantia nigra to DA receptor stimulation following long-term treatment with neuroleptics; this was suggested by recent reports, on the basis of electrophysiological experiments (183,186). The same phenomenon has been suggested with reference to postsynaptic DA receptors in the striatum, in order to explain the tolerance to the effect of neuroleptics on DA metabolism in this brain area (78,83).

The compensatory mechanism could also well be a DA 'auto-receptor' hyposensitivity, resulting from the repeated stimulation by DA released from the nigral dendrites. A similar phenomenon of hyposensitivity has been demonstrated with reference to striatal DA metabolism, following repeated administration of DA receptor stimulants (156). Alternatively, a tolerance to the effect of the inhibitory transmitter (e.g. GABA) probably released by DA from non-DAergic nerve terminals in the pars reticulata (57) may result in a disinhibition of the DAergic cells and the observed increased concentrations of HVA and

DOPAC. Thus, it would appear that the DAergic neurons of the substantia nigra might be self-inhibited under certain circumstances, such as the chronic neuroleptic treatment.

Finally, a rather remote possibility should be borne in mind in relation to the increased HVA and DOPAC in the substantia nigra following chronic treatment with haloperidol, i.e. that the results were misled by the inclusion in the 'substantia nigra' tissue of the DA cell body area A10 which gives rise to the mesolimbic DAergic system (8,9). Since it has been repeatedly shown that no tolerance to the acute effects of neuroleptics on DA turnover develops in the mesolimbic areas of the brain after chronic treatment (71,152), the effects seen in the tissue thought to represent substantia nigra might be partly due to changes in the A10 cell body area.

Further studies are needed in order to clarify the precise mechanisms involved. Measurements of the number of DA receptors, of the activity of DA-sensitive adenylate cyclase, of GABA-receptor binding sites, or of tyrosine hydroxylase activity in the substantia nigra after chronic treatment with neuroleptics, and parallel study of these parameters in the corpus striatum could contribute to elucidation of the specific, functional characteristics of the processes involved in the regulation of DAergic neuronal activity in the nigro-striatal system. Furthermore, inhibition of DA synthesis, e.g. by α-methyl-p-tyrosine, could be a useful procedure for the differentiation of possible pre- or post-synaptic effects of chronic neuroleptic treatment on DA metabolism.

3.4.3 Effects of chronic treatment with neuroleptics on the cholinergic system

In addition to the possibility that tolerance to the prolonged administration of neuroleptics results from changes in the dopaminergic system itself (adaptation phenomena, such as receptor supersensitivity) other neuronal systems which may control or interact with the DAergic system might be involved. Several investigators found that repeated administration of haloperidol produced effects on the cholinergic and GABAergic systemsin both striatum and nigra, which were markedly different from those obtained after a single injection (173, 202, 155). These neuronal systems are believed to have synaptic contacts with the nigro-striatal DAergic pathway, at a pre- or post-synaptic level, in both corpus striatum and substantia nigra (Section 2, 2.1.3 and Fig. 2.2).

The present study demonstrated that after prolonged administration of neuroleptics, changes at a pre- or post-synaptic level in the DAergic nigrostriatal neurons may occur, which are distinct from the effects of acute administration. Thus, in agreement with reports in the literature, the cholinergic and the GABAergic systems were found to be affected in a manner frequently correlating with the changes in the DAergic system.

A single injection of 1mg/kg i.p. haloperidol probably increased the activity of the cholinergic neurons in the striatum, as the increase in CAT activity indicates. Doses of 5 or 10 mg/kg of this neuroleptic had no significant effect on CAT activity. In the substantia nigra, a single dose of 1 mg/kg of haloperidol did not change the activity of the enzyme, although higher doses marginally decreased it (Fig. 3.1).

A similar response of the cholinergic system to a single administration of haloperidol was obtained by other investigators using other parameters as indices of cholinergic activity, e.g. Ach turnover time (173) or Ach concentrations (155). Conversely, acute amphetamine decreased the CAT activity in the striatum (Table 3.5) From these and from the results of the present study (as well as the results of Section 2, Fig. 2.1) it can be inferred that the DAergic pathway is inhibitory on striatal cholinergic neurons. Acute blockade of the postsynaptic DA receptors in the striatum may release these neurons from the inhibitory influence and cause an increase in their activity. The known anatomical or biochemical data do not seem to offer a simple explanation of the effects on the CAT activity in the substantia nigra, which were probably not statistically significant. The finding that higher doses of the neuroleptic did not enhance the effect of the moderate dose (1 mg/kg) in either of the two structures, could be due to inhibition of the enzyme by higher concentrations of haloperidol; this was suggested by the results of in vitro experiments (Nicolaou, unpublished), which showed that haloperidol may significantly reduce the activity of CAT in striatal homogenates at concentration 4×10^{-4}M. Therefore, the possibility that the effects of chronic haloperidol on the cholinergic system are merely effects of cumulative amounts of this drug should not be totally discounted.

The effects of acute administration appeared to be different or were reversed by the chronic treatment with haloperidol (Fig. 3.1). Thus, after 15 daily injections, a slight but not statistically significant decrease, compared to the control levels, was obtained in striatal CAT activity. The CAT activity in chronically treated animals was, however, significantly lower than the activity in animals

treated acutely. The activity of CAT in the substantia nigra was not different from control 1 hour after the last daily injection of haloperidol. However, a marked increase in the nigral CAT activity was found 1 day after the termination of treatment with haloperidol, which slowly returned to normal values. These results are consistent with previous studies which showed a normalisation of Ach concentrations in the striatum (155) or of Ach turnover time in the striatum and the substantia nigra (173) following chronic administration of various neuroleptics, contrary to the acutely-induced marked effects.

The normalisation of CAT activity in the striatum following sustained blockade of DAergic transmission by haloperidol correlates with the tendency of the DA metabolite concentrations to return to baseline level and it is compatible with the DAergic neurons being inhibitory on striatal cholinergic interneurons. Similarly, the elevation of CAT activity in the substantia nigra, which coincides with an increase in the DA metabolite concentrations following chronic administration of haloperidol, is compatible with the proposed excitatory - modulatory influence of cholinergic interneurons on DA cell activity in the substantia nigra. The same conclusion could be drawn from the finding that apomorphine and amphetamine, two drugs known to cause a reduction of DAergic cell firing rate, produced significant inhibition of the CAT activity in the substantia nigra (Table 3.5).

The diminution of the effects of acute treatment on CAT activity after chronic treatment with neuroleptic drugs suggests that tolerance to these effects may develop. Tolerance to the effects of neuroleptics on striatal cholinergic activity also suggests that certain EPS may be related to altered Ach synthesis, release and

metabolism, since these effects (which are thought to be due to excessive striatal cholinergic activity)(188) gradually disappear during prolonged treatment (83). Support for this hypothesis comes from studies with neuroleptics, such as clozapine, which does not elicit EPS in man (193) or catalepsy in rats (78) and is a potent cholinergic (muscarinic) receptor blocker (188). The haloperidol-induced EPS disappear on prolonged neuroleptic administration (128). Furthermore, it has been suggested that there is an inverse relationship between the in vitro affinity of antipsychotic drugs for central muscarinic Ach receptors and their propensity to cause extrapyramidal disturbances in man (188). The findings that the antimuscarinic action of clozapine was preserved after chronic treatment, and chronic administration of haloperidol induced an anticholinergic action (173) or resulted in a different effect on cholinergic activity compared to a single injection (present study, (155)), support the hypothesis that cholinergic hyperactivity in the striatum may be responsible for certain EPS. Whether the changes induced in the striatal cholinergic activity are due to changes in the cholinergic neurons themselves or are due to changes in the dopaminergic neurons synapsing on them needs to be further investigated.

The mechanisms of induction or inhibition of enzyme activity by the neuroleptics in particular needs to be studied using different approaches such as in vitro studies. The various possibilities were mentioned in Section 2 (2.4.3). Our finding (unpublished) that haloperidol at doses 4×10^{-4}M causes marked inhibition of CAT activity in striatal homogenates may also explain the inhibition caused by chronic treatment with this drug. Continued exposure of the cholinergic

cells in the striatum, which probably contain part of the DA receptor population, to the neuroleptic may lead to neuronal adaptation, finally resulting in altered synthesis of enzyme (CAT) or simply in altered activity of existing enzyme.

3.4.4. Effect of chronic treatment with neuroleptics on the GABAergic system

The marked differences between the acute and the chronic effects of neuroleptics on GABA concentration or GAD activity found in this study indicate that the state of functional interaction between the DAergic and the GABAergic system may be differently altered by these two treatments. The GABAergic systems in the striatum and in the substantia nigra seem to be involved in the phenomenon of tolerance elicited by the chronic treatment with haloperidol or α-flupenthixol.

A single injection of haloperidol lowered GAD activity in the striatum but not in the substantia nigra, in agreement with a recent report (202). Chronic haloperidol administration significantly increased the GAD activity in both brain regions, as it was found by the same research team (202). The increase of GAD activity persisted after termination of treatment for 3 days in the substantia nigra and for 1 day in the striatum (Fig. 3.1). The finding that GABA concentrationin the striatum was not altered after acute haloperidol but was decreased after chronic administration, compared to the corresponding effects on GAD activity, indicates that the turnover of GABA was probably altered by the chronic neuroleptic treatment. Thus acute treatment appeared to decrease the turnover rate of GABA in the striatum and chronic treatment seemed to reverse this effect,

in agreement with other reports (173, 202).

The GABA concentration in the substantia nigra was not changed after acute treatment but significantly increased after prolonged haloperidol administration. This result conflicts with reports showing that neuroleptics administered acutely reduce the nigral GABA concentration (202, 204). Although the disagreement may be due to the different dosage and time-course or even due to post-mortem changes or dissection procedure differences, a second neuroleptic (α-flupenthixol) produced a similar effect and the DA receptor agonist apomorphine produced the opposite effect. The marked increase of GABA concentration and GAD activity in the substantia nigra after chronic haloperidol administration indicates a persistent increase in the turnover of this aminoacid, as it was found by other investigators (202).

Thus, definite changes in the GABAergic neuronal activity are elicited by chronic neuroleptic treatment. These changes may be related to the onset of the antipsychotic effects or to the disappearance of certain EPS. A comparison of the changes in the DAergic, cholinergic and GABAergic system in the striatum and the substantia nigra shows no correlation and indicates that alternative mechanisms may be initiated during prolonged treatment with neuroleptics. Routes of interaction of the DAergic system with the other two systems, other than those normally functioning, may be triggered by the chronic impairement of DAergic transmission.

The acute reduction of GAD activity in the striatum due to haloperidol does not seem to be critically related to the extent of the anticholinergic properties of this neuroleptic, since clozapine, an antipsychotic with a strong anticholinergic action, produced a similar

effect on GAD (202). Therefore, DA released from nigrostriatal neurons may directly stimulate striatal GABA neurons, probably cell bodies of the striato-nigral GABAergic pathway. Blockade of DA receptors on the GABAergic neurons would result in an inhibition of the activity of these neurons, reflected in a decrease of GAD activity. According to the original 'feedback loop' hypothesis of Carlsson and Lindquist (44), the reduction of the activity of the striatonigral inhibitory neurons leads to an increase in DA cell firing in the substantia nigra and an increase in DA turnover in the striatum. Although this sequence has been disputed (164, 165, 166) a direct contact of the DAergic nerve terminals with the GABAergic neurons may be postulated to exist, alongside the indirect link through cholinergic interneurons. The nature of the direct DA-GABA link in the striatum seems to be stimulatory, as shown by the increase in striatal GAD induced by L-dopa administration (202) or the highly significant increase of striatal GABA concentration by apomorphine (Table 3.5), whereas the inhibitory DA-Ach may be followed by a stimulatory Ach-GABA connection, as shown by changes in GABA levels induced by cholinergic agonists and antagonists (105,106), resulting in an overall inhibitory influence (as depicted in Fig. 2.2).

Bearing in mind these two alternative routes for the interaction of the nigrostriatal DAergic pathway with the GABA-containing neurons in the striatum, we could hypothesise that under certain conditions one of the two mechanisms predominates. Acute blockade of postsynaptic DA receptors may open up the direct link, whereas chronic treatment and the demonstrated tolerance to the effects on DA turnover and Ach turnover may proceed through the indirect route. Therefore, a reversal of the acute inhibition of GAD would be

expected, i.e. an increase of GAD activity. The decrease in GABA concentration after chronic haloperidol might indicate an increase in turnover (release and utilisation of GABA) or an increase in GABA output from the striatum. The latter possibility is supported by the finding that both GAD activity and GABA concentration were increased in the substantia nigra, indicating possible increased GABAergic neuronal activity. The finding that DA metabolite levels were still significantly higher than control in the substantia nigra, while GABAergic activity in this region appeared to be increased, argues against the postulated feedback regulation of the DAergic nigro-striatal pathway by the descending striatonigral GABAergic pathway. In addition, the diminished effects of neuroleptics on DA turnover in the striatum after chronic administration support the existence of local independent mechanisms for the regulation of DAergic activity in the DA cell-body area of substantia nigra and the DA nerve terminal area of corpus striatum. A 'chemical' denervation appears to result from the chronic blockade of DA receptors, and a dissociation of the DA cell-bodies and the DA nerve terminals seems to operate with regard to the regulation of DA metabolism. The effects of chronic neuroleptic treatment on DA turnover in the striatum could simply result from the activation of the intrastriatal GABAergic neurons, postulated to impinge on (and regulate the activity of) the DAergic nerve terminals at a prejunctional level (Fig. 2.2).

The finding that amphetamine significantly increased GAD activity and GABA concentration in the substantia nigra is in agreement with the GABA-releasing properties of this agent, found in nigral slices in vitro (57) and suggests that the neuroleptic-sensitive release of DA from dendrites in the substantia nigra may stimulate in turn the

release of GABA, which could then act to inhibit the DA cell-bodies or the dendrites.

The stimulatory effect of apomorphine on GAD activity in the substantia nigra (Table 3.5) may also support the idea that DA and DA receptor agonists act in this area as GABAmimetics. The reduction of nigral GABA accompanying the increase of GAD activity following this treatment may indicate an increase in release and degradation of GABA.

However, chronic blockade of nigral DA receptors by haloperidol does not lead to a maximum activation of DAergic activity (as the elevated HVA-DOPAC concentrations indicate). The increase in GAD activity and GABA concentration in the substantia nigra after chronic haloperidol may indicate an enhancement of DA receptor stimulation (probably on GABAergic neurons) and consequently of GABAergic activity, which in turn causes an increased inhibition of DA neuronal activity. The latter effect, however, does not seem to explain the finding that DA turnover is still high in the substantia nigra, whereas it could explain the normalisation or the reduction of turnover observed in the striatum. Alternatively, changes in the nature of GABA receptors in the substantia nigra (probably located on DAergic neurons), such as subsensitivity, may result from the continuous stimulation by released GABA leading to a reduced inhibition of the DA neurons and a consequent increase in DA turnover.

3.4.5 Conclusions

In conclusion, comparison of the effects of chronic neuroleptic treatment on the three neuronal systems demonstrates that functional links may operate between the three systems in both substantia nigra

and striatum. The development of tolerance to the effects of neuroleptics on DAergic activity in the striatum does not seem to be accompanied by a readjustment of the firing rate of the DAergic cells in the substantia nigra. Tolerance to the effects of neuroleptics on the cholinergic activity in the striatum (which are normally excitatory) seems to follow, probably as a secondary phenomenon, after the chronic blockade of DA receptors. Changes in the DA receptor itself or in the number of striatal DA receptors (as has been reported) may be the primary stage in the development of tolerance. Otherwise, the effects of neuroleptics on the cholinergic or the GABAergic system subsequent to the DA receptor blockade may, in the form of local feedback regulatory processes, contribute to the readjustment of the DAergic activity. Chronic indirect activation or deactivation of the GABAergic or cholinergic interneurons, thought to impinge on DAergic terminals at a prejunctional level, may result in adaptation of DAergic neuronal activity, in changes of the DA receptor itself or of the number of receptors and in the demonstrated increase of the sensitivity of DA receptors to direct stimulants. The links probably existing at physiological states between the DAergic terminals and the GABAergic neurons (excitatory), between the DAergic terminals and the cholinergic neurons (inhibitory), and between the cholinergic and the GABAergic neurons (excitatory) (Fig. 2.2) do not seem to operate in the same way after acute and chronic treatment with neuroleptics.

The chronic treatment provided further evidence for the reciprocal relation of DA turnover between the striatum and the substantia nigra. In contrast to the tolerance observed in the striatum, the activity of the DAergic neurons in the substantia nigra and the consequent increase of HVA and DOPAC in this area did not show

any adaptive response after chronic neuroleptic administration. Moreover, the proposed DA-GABA interaction in the substantia nigra, as evidenced by the GABAmimetic action of apomorphine and amphetamine and the anti-GABAmimetic action of acute haloperidol (Section 1, 1.4.2.5) did not appear to occur after chronic blockade of DAergic transmission.

Further experiments are needed in order to establish the nature of the primary effects of chronic neuroleptic treatment in the substantia nigra. An investigation of the effects on the characteristics of the DA receptor and the DA-sensitive adenylate cyclase associated with it (which has been found in substantia nigra), an evaluation of any changes in the number of DA receptors (as it has been found in the striatum) and any consequent DA receptor supersensitivity phenomena, could further help in elucidating the mechanism of tolerance to the chronic administration of neuroleptics.

3.4.6 Lesion- and drug-induced 'supersensitivity' and relevance to Parkinsonism

Animals treated chronically with neuroleptic drugs displayed an enhanced sensitivity to DA receptor stimulants (72,78). A similar 'supersensitivity' was apparent when DAergic transmission along the nigrostriatal neuronal pathway was interrupted by lesions of the substantia nigra (8,110). The common feature in these phenomena, i.e. the prolonged blockade of DA neurotransmission along the nigrostriatal pathway, is present in certain pathological states in humans, such as Parkinson's disease. The characteristic substantial loss of DA cells in the substantia nigra of Parkinsonian patients (117) and the specific destruction by 6-OHDA of the DA-containing cells in the same structure in rats (150) have been postulated to represent a

similar phenomenon (110). Several biochemical and behavioural findings elicited by the various states of chronic blockade of DAergic transmission, those involving the cholinergic and the GABAergic system in particular, demonstrate profound similarities. A comparative study of these findings may be useful in defining the interrelation of the various neuronal systems.

Degeneration of the DA cells in the substantia nigra of Parkinsonian patients and in the substantia nigra of rats injected with 6-OHDA directly into this area, is characterised by a marked decrease in the concentration of DA and its synthesizing enzymes in the substantia nigra (117,8). In addition to this main neurochemical effect, there are other consistent alterations of other neurotransmitter systems in the substantia nigra (Table 3.6). Among these, the alterations related to the GABA-containing neurons are of particular interest. A decreased functional state of the nigral GABA neurons is implied by the reduction in the concentration of GABA and the GAD activity in animals with 6-OHDA-lesions (Section 2) and by the diminution of GAD activity in Parkinson's disease (202,206). This might be interpreted as meaning that an atrophy or degeneration of the GABAergic nerve terminals in the substantia nigra follows the DA cell degeneration. The proposed synaptic contacts and the mutual regulation of neuronal activity are consistent with these data. A biochemical inactivity of GABA neurons subsequent to an impaired DAergic influence is also manifested in the reduction of the DA-sensitive adenylate cyclase activity in the substantia nigra after lesions of the striatonigral GABAergic neurons (56,58) and by the reduction of GABA-binding sites in Parkinsonian patients specifically in the substantia nigra (202). Chronic neuroleptic treatment, however,

Table 3.6

Changes in glutamic acid decarboxylase (GAD) and Choline acetyltransferase (CAT) activities and GABA concentration in the corpus striatum and the substantia nigra of parkinsonian patients and of rats with 6-hydroxydopamine (6-OHDA)-induced lesions in the substantia nigra or treated for 15 days with the neuroleptic drug haloperidol.

	substantia nigra				corpus striatum			
	CAT	GAD	GABA	DA turnover	CAT	GAD	GABA	DA turnover
Parkinson's	↓	↓	↓	↓	↓	↓	—	↓
6-OHDA	↓	↓	↓	↓	↑	↑	↑	↓
Chronic neurol.	↑	↑	↑	↑	—	↑	↓	↓

Data for parkinsonian brains are from refs. 202, 206, 117.

Data for animal experiments are from Sections 2 and 3 of the present Thesis.

The arrows indicate the direction of change, i.e. ↓ : significant decrease; ↑ : significant increase; — : no significant change.

did not lead to DAergic inactivity in the substantia nigra, but to an increase in turnover (Fig. 3.1). The same concept of DA-GABA interaction seems to explain the consequent increase in nigral GABA turnover (Table 3.6, 173).

Similarly, the cholinergic neurons in the substantia nigra seem to receive a DAergic 'trophic' influence, since reduction of DA results in a reduction of CAT activity in both Parkinson's disease (206) and in 6-OHDA lesions (Section 2). On the contrary, the increase in DA turnover after chronic neuroleptic treatment, probably results in an increase in CAT activity (Table 3.6).

In the striatum, a reduction of DA is the common feature in Parkinsonian patients (117) and in rats lesioned with 6-OHDA in the substantia nigra (Section 2). Chronic neuroleptic treatment also leads to a reduction of DA turnover compared to the acute treatment (Fig. 3.1). The reduction of the known inhibitory influence of DAergic nerve terminals on the striatal cholinergic interneurons after 6-OHDA lesions results in an increase in striatal cholinergic activity. The normalisation of DA turnover is followed by a normalisation of the CAT enzyme activity (Table 3.6). In Parkinsonian brains, however, a 50% reduction of CAT activity in the striatum was found (206) probably indicating biochemical inactivity of the cholinergic neurons following the degeneration of the DAergic neurons which directly synapse with them. In this respect, an important difference is noted between Parkinson's disease and the proposed animal model for this disease.

The possible importance of GABA and GAD in Parkinson's disease is not clearly known. However, areas such as substantia nigra and globus pallidus, which show characteristic pathological changes in brains of Parkinsonian patients, were found to have selectively decreased GAD enzyme activities (202,206). The striatal GAD activity

was also found to be reduced (202,206) whereas the GABA concentration did not show any apparent change (202). Thus, there might be a decrease of GABA turnover or a decrease of synthesis and also of release of GABA in the striatum, subsequent to the chronic degeneration of DAergic neurons. Acute treatment with neuroleptics was also found to produce a reduction of GAD activity but prolonged blockade of DAergic neuro-transmission by these agents increased GAD activity and reduced GABA concentration (Table 3.6).

The difference between the effect of acute and chronic neuroleptic treatment and Parkinson's disease, indicate that an alternative functional interrelation of DAergic and GABAergic neurons in the striatum may be unmasked after prolonged impairment of DAergic neurotransmission. Indeed, as it has been discussed in detail in Section 2, more than one way seems to exist in which the DAergic and the GABAergic neurons influence each other's activity. Therefore, degeneration of the DAergic terminals may have different effect on GABAergic neurons synapsing with them in the striatum than the prolonged blockade of DA neurotransmission by chronic treatment with DA receptor antagonists.

Lesions with 6-OHDA in the substantia nigra of rats also seem to produce effects on striatal GAD and GABA which do not appear to be comparable to the effects of Parkinson's disease. Thus, an increase in GAD activity and GABA concentration in the striatum of rats lesioned in the substantia nigra with 6-OHDA was found, in contrast to the decrease in GAD and lack of effect on GABA found in Parkinson's disease or the increase in GAD but decrease in GABA that results from chronic neuroleptic treatment (Table 3.6). The involvement of cholinergic interneurons between the DAergic terminals and some GABA-

ergic neurons in the striatum (105, 106) and the different effect of the three syndromes under study on CAT activity (Table 3.6) indicate that these cholinergic interneurons may mediate part of the effects of DA on GABA neurons. A comparison of the effects of 6-OHDA and Parkinson's disease, in particular, shows that a positive correlation exists between CAT and GAD activity in the striatum, which suggests that the cholinergic neurons may exert a trophic influence on the GABAergic neurons, perhaps mediated by a stimulatory action of Ach at receptor sites located on GABAergic neurons.

The major neurological manifestations of Parkinsonism are akinesia, tremor at rest, rigidity and loss of postural reflexes (177). A relationship between central cholinergic overactivity and Parkinsonian tremor has been demonstrated in patients (179) and in laboratory animals (210). The tremorgenic action of cholinomimetics in rats was increased by treatment with neuroleptics (211). In dogs destruction of catecholamine-containing neurons by 6-OH-DA produced Parkinsonian signs which were aggravated by doses of cholinomimetics that had no effect on control animals (212). Tardive dyskinesia resulting from chronic treatment with neuroleptics has been postulated to be due to striatal DA receptor supersensitivity (78). Stimulation of these receptors by DA receptor agonists also resulted in dyskinesias in animals pretreated with 6-OHDA (213) as it did in patients with Parkinson's disease (212). Treatment of tardive dyskinesia is based on the hypothesis that DAergic hypersensitivity and resultant cholinergic hypoactivity in the striatum is responsible for the symptomatology.

These pharmacological and clinical data demonstrate the involvement of striatal cholinergic activity in certain extrapyramidal

side effects and offer clues to possible therapies. However, the alterations in the GABAergic activity, their importance in the manifestation of these symptoms and possible treatments aiming directly at restoring the normal GABAergic function have not been studied. The evaluation of the activity of GABAergic neurons following DAergic deficiency may enable the elucidation of the mechanism of the underlying neurological disorders and suggest alternative or supplementary treatments to those currently employed.

SECTION 4

EFFECT OF L-TRYPTOPHAN AND MONOAMINE OXIDASE INHIBITORS ON CATECHOLAMINE METABOLISM IN THE RAT BRAIN

4.1 INTRODUCTION

4.1.1 5-Hydroxytryptamine metabolism and storage in brain

The principal metabolic pathway of 5-hydroxytryptamine (5-HT) is shown in Fig. 4.1. As the blood-brain barrier is impermeable to the amine (214), it must either be synthesised from tryptophan in the brain or from 5-hydroxytryptophan (5-HTP) taken up from the blood. 5-HTP however is normally undetectable in rat plasma (215), therefore the hydroxylation of tryptophan possibly takes place in the brain itself. As the evidence described below shows, the amine could be synthesised in the nerves where it is found.

Tryptophan is converted to 5-HTP by the enzyme tryptophan-5-hydroxylase; its presence in brain has been shown by several groups (216,217). The regional distribution of the enzyme was shown by Peters et al.(218) to parallel the distribution of both 5-HT and 5-HT-containing nerve terminals (as described by Dahlström and Fuxe, ref 5). 5-HTP is decarboxylated to 5-HT by 5-HTP decarboxylase, an enzyme widely distributed in the brain (219). The hydroxylation is the rate limiting step in the synthesis of 5-HT; Ichiyama et al.(217) have shown that, in vitro, the decarboxylase activity is far in excess of the hydroxylase, and, in vivo, Moir and Eccleston (220) reached the same conclusion from studies on the administration of 5-HTP or L-tryptophan. The latter report, as well as several others also indicate that the rate of hydroxylation is highly dependent on the concentration of tryptophan in the brain (215), which in turn depends on the plasma unbound tryptophan level (221).

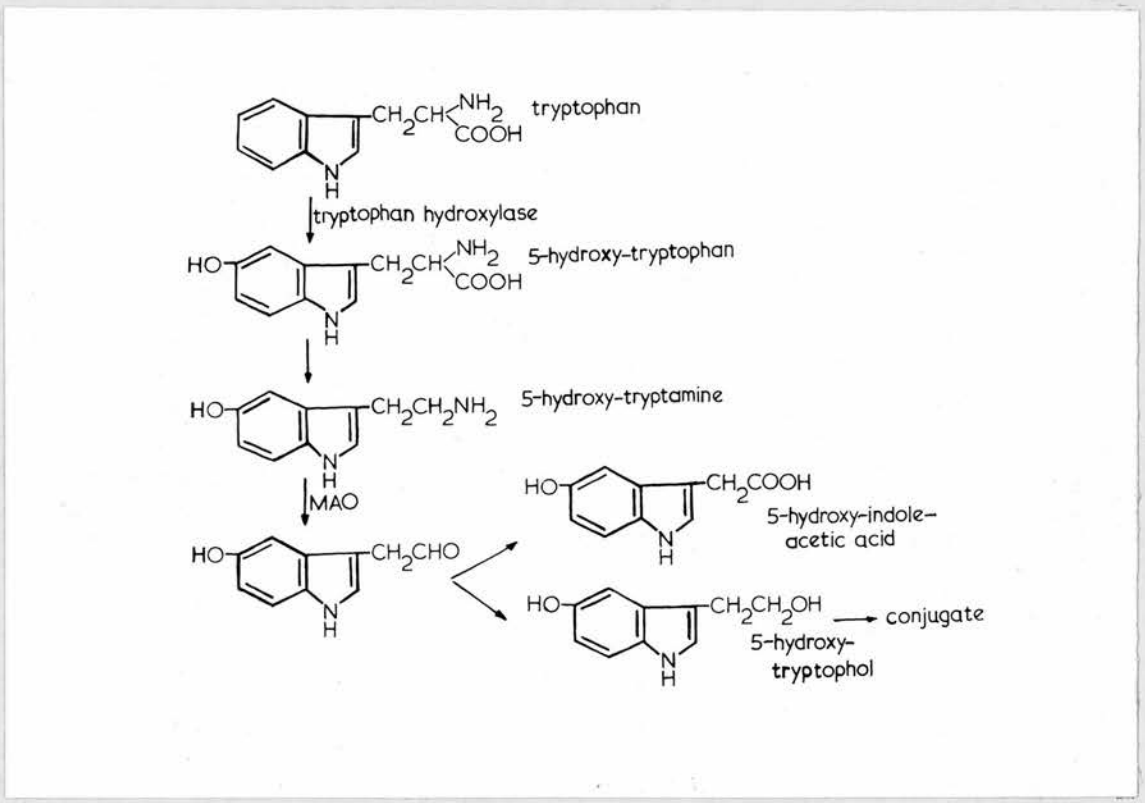


Fig. 4.1

Principal metabolic pathway of 5-hydroxy-tryptamine (5-HT) in brain.

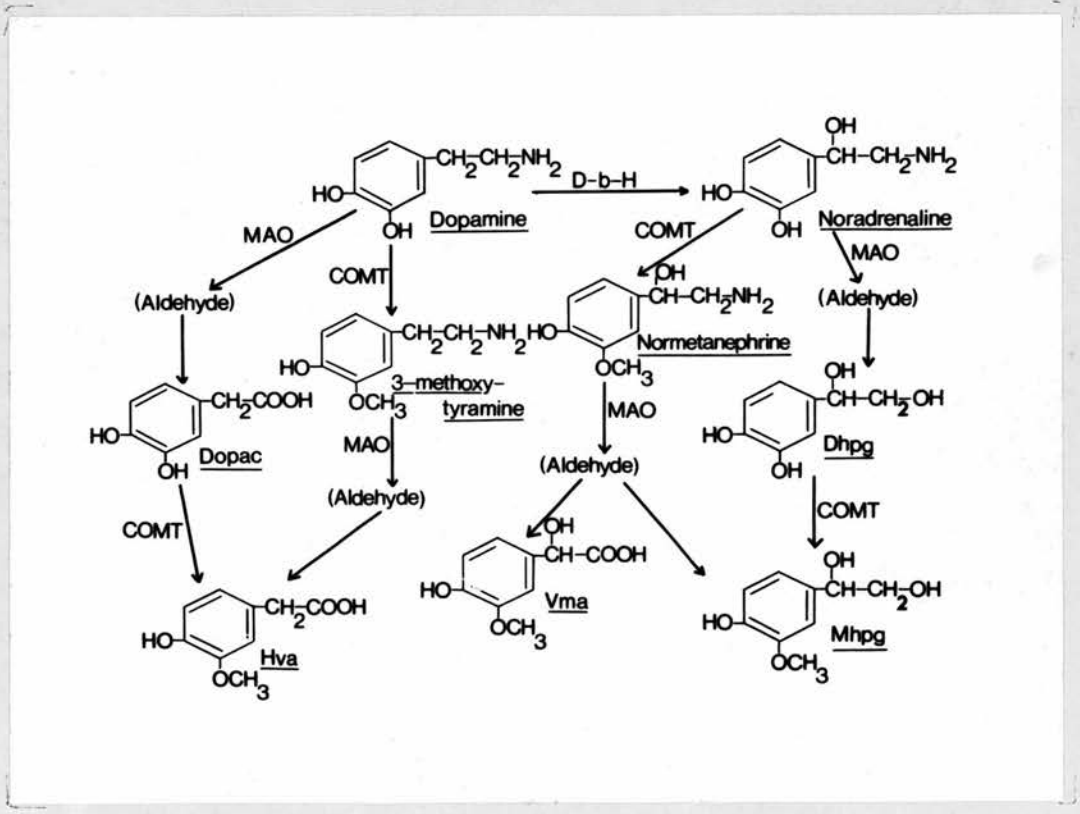


Fig. 4.2

Principal metabolic pathways of dopamine and noradrenaline in brain.

Theoretically, 5-HT could also be synthesised by decarboxylation of tryptophan to tryptamine and then hydroxylation. However, due to the relative affinities of the alternative substrates for the enzymes (217), this pathway is not important.

5-HT is catabolised by monoamine oxidase (MAO) to 5-hydroxy-indole acetaldehyde and then mostly to 5-hydroxyindol-3-yl acetic acid (5-HIAA) by aldehyde dehydrogenase. Some of the aldehyde may be converted to 5-hydroxytryptophol by alcohol dehydrogenase, as has been shown in brain slices in vitro (222).

By the use of fluorescence microscopy, Dahlström and Fuxe have shown that 5-HT in brain is largely concentrated in nerve endings (5). Subcellular fractionation of brain homogenates by Michaelson and Whittaker (223) showed that a substantial fraction of the 5-HT in brain is associated with pinched-off nerve endings or 'synaptosomes'. This was confirmed by Aghajanian and Bloom (224), who used autoradiography to localise intraventricularly injected (^{3}H)-5HT; they found most of the radioactivity in nerve terminals and unmyelinated axons. In addition, they noted that most of the terminals which showed activity contained so-called dense-core vesicles, which suggests that 5-HT may be stored in such vesicles in the nerve endings.

Subcellular fractionation studies by Green and Sawyer (216) showed that 40 to 60 per cent of the tryptophan hydroxylase was present in the 'crude mitochondrial' fraction. Further fractionation into synaptosomal and mitochondrial fractions (217) indicated that most of the activity of the crude mitochondrial fraction was contained in the synaptosomes rather than in the mitochrondria. 5-HTP decarboxylase is a soluble enzyme and its activity is distributed throughout the different subcellular fractions (217). Monoamine oxidase is thought

to be located exclusively in the mitochondria (225).

4.1.2 Catabolism of catecholamines in brain

The principal metabolic pathways of DA and NA are shown in Fig. 4.2.

Mannarino et al.(226) in 1963 described the formation of normetanephrine, 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) and a small quanitity of vanillyl mandelic acid (VMA) in the brain of the cat after the intraventricular administration of (^{14}C)-NA. In the rat brain, Glowinski et al.(227) found that (^{3}H)-NA injected into the lateral ventricle is metabolised to normetanephrine, is deaminated and, also, forms O-methylated deaminated metabolites. Studies with slices of rabbit brain in vitro (228) showed that NA is metabolised mainly to the two glycols MHPG and dihydroxyphenyl glycol (DHPG). Much less is known of the metabolism of endogenous cerebral NA. The two glycol metabolites MHPG and DHPG have been shown to be present in brain tissue (229). In some species, such as rat and man, MHPG is further metabolised in the brain to its sulphate conjugate (229).

Studies with slices of rabbit brain in vitro (228) showed that radioactive DA is converted mainly to DOPAC and HVA, as well as to a small amount of 3-methoxytyramine. DOPAC (230), HVA (231,232) and also 3-methoxytyramine (233) were identified as being present in normal brain tissue. A more detailed reference to DA metabolism is made in Section 1.

4.1.3a Tryptophan loading

Tryptophan loading has been used as a means of determining the rate of 5-HT metabolism. It consists of injecting the animal with a large dose of L-tryptophan, so that tryptophan hydroxylase becomes saturated by its substrate, and the resultant increases in 5-HT and

5-HIAA can be measured. The pattern of metabolites is the same as under normal conditions (220).

Ashcroft et al. (215) showed that after rats had received a large dose of L-tryptophan (800 mg/kg i.p.), 5-HT increased to a maximum after 1 hour, and 5-HIAA was maximal after 4 hours. Grahame-Smith (234) demonstrated that with doses of L-tryptophan up to 120 mg/kg i.p. the amount of 5-HT accumulating in the rat brain within 1 hour was dependent upon the brain tryptophan concentration.

Tryptophan loading can give a dynamic profile of the whole metabolic pathway, i.e. it indicates whether a certain experimental situation is acting on the synthesis or breakdown of 5-HT or on the excretion of 5-HIAA from the brain. The disadvantage is that a rather unphysiological situation is produced. The level of tryptophan in the brain is many times normal (234) and the saturation of the pathway may upset the regulatory mechanisms, e.g. Aghajanian (235) has found a reduction in the firing rates of 5-HT-containing cells in the raphe area after administration of L-tryptophan; as with MAO inhibition (236), the reduced firing rate is probably linked to the elevated 5-HT concentration.

4.1.3b Monoamine oxidase inhibition

The rate of accumulation of 5-HT after inhibition of MAO has been used to measure its rate of synthesis. After administration of a MAO inhibitor, 5HT in the brain builds up linearly for about an hour and eventually plateaus at around three times the normal level (237). That plateau is reached probably indicates either a loss of the amine by diffusion, or a feedback inhibition of its synthesis (end-product inhibition). Aghajanian et al. (236) found that a variety of MAO inhibitors depress the firing rates of raphe cells, the effect

appearing gradually over a period of about 30 min. This was possibly due to the accumulation of 5HT, as there was no depression of firing after previous depletion of 5-HT with p-chlorophenylalanine.

No marked hyperactivity was observed after MAO inhibition (234).

4.1.3c Combination of tryptophan loading with monoamine oxidase inhibition

When L-tryptophan was administered to rats, there was a small increase in brain 5-HT concentration, but a large increase in the concentration of the 5-HT metabolite, 5-HIAA, indicating an increase in the rate of synthesis of the amine, which was then broken down by intraneuronal MAO; no gross behavioural changes occurred. When the rats were treated with a MAO inhibitor followed by L-tryptophan at a dose level which produced submaximal increases in brain 5-HT, motor behaviour was strikingly increased (234), probably due to a spillover of 'active' 5-HT on to postsynaptic receptors. Appearance of this '5-HT-induced' hyperactivity syndrome depends on MAO inhibition, tryptophan administration and tryphophan hydroxylase and 5-hydroxytryptophan decarboxylase activity (since inhibition of either of these two enzymes abolished the hyperactivity) (234) but the precise mode of action of this combination is not clear. There is a positive correlation between the rate of accumulation of brain 5-HT and the rate of development of the hyperactivity, which suggests that the syndrome could be produced by 5-HT receptor stimulation (238). However, it is probably not just the excess 5-HT which is responsible for hyperactivity, as there was no behavioural change after the administration of a MAO inhibitor or tryptophan alone (234). It has been also suggested that even in normal conditions 5-HT is in excess of that required to fulfill the functional needs of the brain (239),

the excess 5-HT being contained by the intraneuronal pools (one of which is identified as vesicular binding) or metabolised by intraneuronal MAO. When MAO was uninhibited, L-tryptophan did not produce hyperactivity, suggesting that normally both the granular and the cytoplasmic MAO act together to regulate the size of the prefunctional pools (240).

4.1.4 Interaction of 5-hydroxytryptamine with the catecholamines

There have been several reports that 5-HT can be taken up into catecholamine-containing neurons. (^{3}H)-5-HT is taken up at very low concentrations (4.4×10^{-8}M) not only by serotonergic (i.e. 5-HT-containing) but also by DAergic terminals, as has been shown in an in vitro preparation of rat striatal homogenates (241). Amphetamine has been shown in vitro to release ^{3}H-5-HT from rat striatal or cortical slices, but at concentrations higher than those required for the release of (^{3}H)-DA or (^{3}H)-NA, e.g. ED 50 for catecholamines $10^{-6} - 10^{-5}$ and for 5-HT 10^{-4}M (242).

Administration of L-dopa (the precursor of both NA and DA) to rats (plus a peripheral decarboxylase inhibitor) induced a decrease in brain 5-HT and an increasein 5-HIAA, due possibly to displacement of endogenous 5-HT from its stores (243,244,245). However, the depleting action of L-dopa on 5-HT storage is present, regardless of whether the catecholaminergic neurons are damaged (246), indicating an indirect action of L-dopa on the metabolism and/or storage of brain 5-HT. A similar phenomenon was found with regard to the effect of L-dopa on brain tryptophan (246). The decarboxylation of 5-HTP and L-dopa is apparently performed by the same enzyme (247) and loading with L-dopa may cause competitive inhibition of the enzyme. Conversely, loading doses of 5-HTP resulted in 5-HT-

derived fluorescence in catecholamine neurons previously depleted of their transmitters (248,267). Therefore, administration of either the precursor of 5-HT or of the catecholamines could result in non-specific stimulation of the other system, due probably to displacement from the neuron and release into the synaptic cleft.

It has been observed in many studies with psychotropic drugs that both DA and 5-HT metabolism may be affected; chlorpromazine, for example, which is believed to block DA receptors (30), inhibited the tryptophan-induced hyperactivity without altering the rate of 5-HT accumulation (249). Depletion of brain DA inhibited the hyperactivity resulting from the administration of a MAO inhibitor together with either tryptophan or 5-methoxy-N,N-dimethyltryptamine, a 5-HT receptor agonist (250). Administration of L-tryptophan, on the other hand, caused a large rise in the concentration of HVA in the cerebrospinal fluid of the dog (251).

All the above evidence points to the existence of an interaction between the cerebral metabolism of 5-HT and DA or NA. It has been postulated that either a group of DAergic neurons lies between the 5-HT neurons responsible for initiating the hyperactivity response and those mechanisms responsible for its behavioural expression (250), or a group of DAergic neurons regulates the magnitude of the behavioural response after the activation of postsynaptic 5-HT receptors (252).

The postulated interaction of DA and 5-HT in the production of the hyperactivity syndrome in rats (250,256) and in a variety of psychological and neurological disorders (255) poses the question whether in certain psychiatric states some kind of imbalance exists between neuronal systems containing these neurotransmitters.

4.1.5 Statement of the problem - purpose of the study

The experiments described in this Section were designed to determine the effects of tryptophan loading on neuronal systems containing DA or NA in brain. The experiments were based on the hypothesis that tryptophan loading results in displacement of the catecholamines from their neurons and consequently elicits biochemical and behavioural responses similar to those obtained after the administration of the monoamine-releasing agent amphetamine. Acute and chronic administration of monoamine oxidase inhibitors were combined to tryptophan loading and the effects on the DA and NA metabolite concentrations assessed and compared to the effects of acute administration of amphetamine.

The investigation of the pharmacological profile of the combination of L-tryptophan plus a MAO inhibitor could offer an explanation of the reported beneficial effect of this treatment in human depression (254) and offer clues to possible improved therapies for affective disorders.

4.2 MATERIALS AND METHODS

4.2.1 Reagents - Drugs

All reagents and solvents were of analytical grade. Distilled water was used throughout. The following drugs were used: L-tryptophan, 5-hydroxytryptamine (serotonin creatinine sulphate) and 5-hydroxyindoleacetic acid, purchased from Koch-Light Lab.; phenelzine sulphate (β-phenylethyl hydrazine) from William Warner and Co.; Amberlite CG50 resin, type 1, 100-200 mesh and benzene hexachloride from BDH; 3,4-dihydroxyphenylacetic acid (DOPAC), bis-(3-methoxy-4-hydroxy-phenyl glycol) piperazine salt (MHPG), tryptamine hydrochloride and DL-amphetamine sulphate from Sigma Chem. Co.; 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA) and trifluoracetic anhydride from Aldrich Chem. Co.; pargyline hydrochloride from Abbott Lab.; helicase enzyme from Micro-Bio Labs.

Before use, the Amberlite CG50 resin was treated as follows: It was first stirred for 30 min with 3 vol. 1M HCl, the acid was decanted off and the resin washed by decantation with distilled water until no chloride could be detected in the supernatant fluid on addition of a few drops of 2.5% ($W/_V$) silver nitrate solution. The resin was stirred twice with 3 vol. 3M NH_4OH for 30 min. The NH_4OH was decanted off and the resin washed repeatedly with distilled water until the pH of the supernatant fluid was 9. The resin was finally treated with 0.2M ammonium acetate buffer pH7.5, until the buffer pH remained unchanged after standing in contact with the resin for at

least 12 hours. The resin was stored under this buffer solution.

4.2.2 Animals

Male Albino Wistar rats, weighing between 200-250 g were used, housed in groups of 3-4 and fed with their normal diet.

4.2.3 Drug treatments

Acute treatment. Phenelzine sulphate (20 $mg/_{kg}$ i.p.) was injected 1 hour prior to the administration of L-tryptophan (100 $mg/_{kg}$ i.p.) and the rats were killed 1 hour after the latter injection. Saline solution (0.9 $g/_{l}$ NaCl) was administered in both cases to the control animals or instead of one of the two drugs when the animals were injected with either L-tryptophan or phenelzine alone.

A suspension of L-tryptophan was administered, prepared as described by Ashcroft et al.(215): the L-tryptophan powder was mixed and ground in a mortar with 3-4 drops of Tween 80 and the saline solution was added drop by drop with continuous grinding, until a homogeneous white suspension was obtained.

Phenelzine sulphate was dissolved in sodium phosphate buffer pH 7.0 for injections, because its aqueous solutions were acidic (pH 2.3) and the injections painful to the animals.

DL-Amphetamine sulphate (16 $mg/_{kg}$ i.p.) was administered to the animals acutely 1 hour before killing.

Pargyline hydrochloride (50 $mg/_{kg}$ i.p.) was injected 2 hours before killing.

Chronic treatment. Phenelzine sulphate (10 $mg/_{kg}$ i.p.) was administered daily, at about 10am., for 15 days. One hour after the last injection, L-tryptophan (100 mg/kg i.p.) or saline solution was injected and the animals killed 1 hour later. Saline solution was administered daily

to animals that served as controls.

4.2.4 Dissection of brain regions

The rats were killed by a blow on the head, and after decapitation, the brains (including cerebellum) were quickly removed, weighed and placed on dry ice (for whole brain biochemical analyses) or on a cold plate for dissection of the brain regions, according to the technique described in Section 1 (1.2.1). The areas corpus striatum, nucleus accumbens and olfactory tubercle were dissected out and analysed on some occasions in the present study.

4.2.5 Biochemical determinations

For biochemical analyses each brain was homogenised in an all-glass homogeniser in 10 ml of 0.4M perchloric acid. The precipitated proteins were sedimented by centrifugation at 2,500 Xg for 15 min at 4°C. The supernatant fluid was transferred to a beaker and the pH adjusted to 7.5 (glass electrode) by dropwise addition with constant stirring, of 5M and then 0.2M KOH. The contents of the beaker were transferred into large (20 ml) centrifuge tubes and centrifuged at 2,500 xg for 5 min at 4°C. The supernatant fluid was separated from the precipitated potassium perchlorate and passed through a weak cation exchange resin (Amberlite CG50, type 1, 100-200 mesh) which had been previously washed with 5 ml of 0.02M ammonium acetate buffer pH 7.5. (Before use the resin was treated as described in 4.2.1).

The procedure of separation of the amines, the metabolites, etc. to be analysed is outlined in Fig. 4.3.

A pasteur pipette (0.5 cm internal diameter) was used as

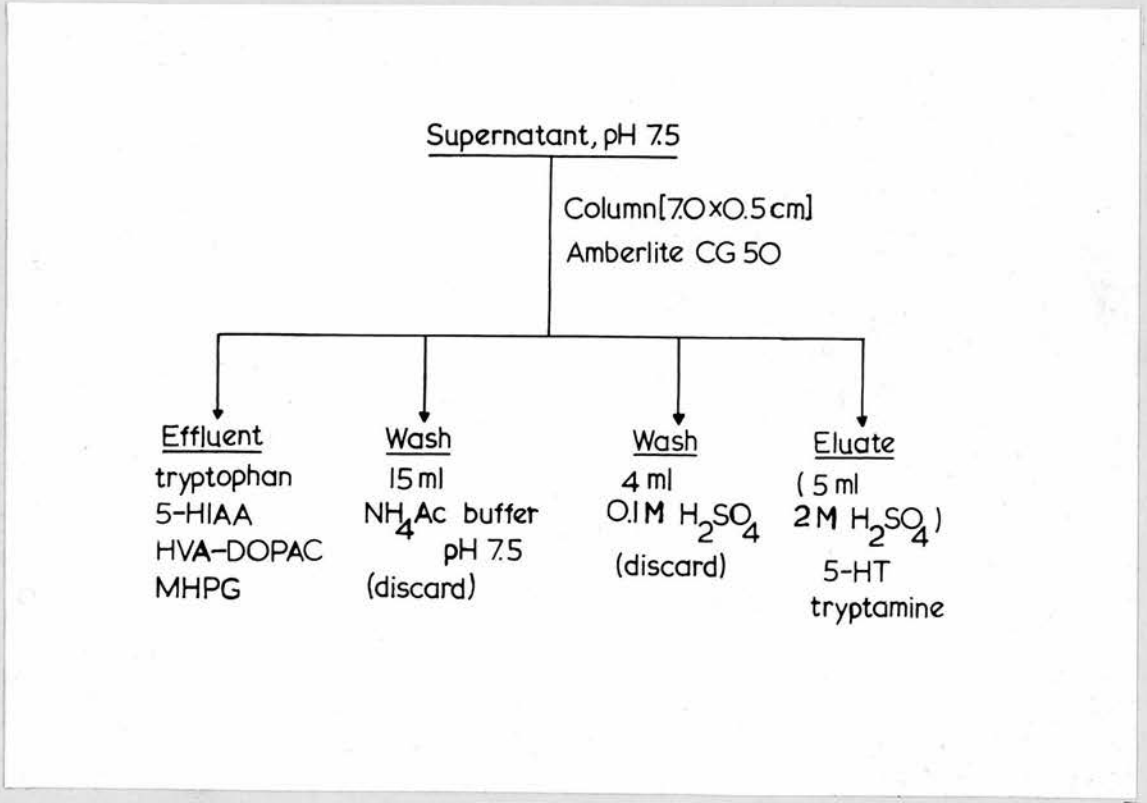


Fig. 4.3

Flow diagram for the separation of tryptophan, acid metabolites and amines from the supernatant fluid obtained after homogenisation of the rat brain and precipitation of proteins.

column, filled with resin (7 cm) and the narrow end was fitted with a plastic tube and a clip in order to control the flow rate through the column. Under the conditions employed, i.e. the pH of the sample solution and the previous treatment of the resin, the amines were absorbed on the resin; the acidic products were not absorbed; the neutral products (such as tryptophan) were either not or were loosely absorbed, and hence easily eluted.

The supernatant was passed through the column at approximate flow rate 10 drops/min and the effluent kept for the assays of tryptophan and the acidic metabolites of 5-HT, DA and NA. Then 15 ml of 0.02 M ammonium acetate (NH_4Ac) buffer, pH 7.5, followed by 4 ml of 0.1M H_2SO_4 were passed through the column at approximately 10 drops/min and the effluent discarded. The amines were eluted from the column with 5 ml of $2MH_2SO_4$ at a flow rate of 5 drops/min, all of the eluate being collected.

The effluent (10-11 ml) was collected, the volume measured accurately and divided for the assays of the acidic or neutral products: Tryptophan, 5-HIAA, HVA, DOPAC and MHPG. The eluate (5 ml) was measured accurately and divided for the determination of 5-HT and tryptamine.

a. Estimation of tryptophan in the column effluent

Tryptophan was determined in less than 1 ml of the column eluate by the 'Norharman' procedure (257). 0.1-0.5 ml of the effluent was diluted to 2.8 ml with 2M H_2SO_4, mixed with 0.1 ml of 18% ($W/_V$) formaldehyde and 0.1 ml of 5% ($W/_V$) hydrogen peroxide, and the mixture was heated in a boiling water bath in a tightly covered test tube for 20 min. The tubes were then cooled down with running water and the fluorescence intensity of the norharman derivative

formed from tryptophan was determined using a Perkin-Elmer MPF3 spectrophotofluorimeter. The norharman derivative showed maximum fluorescence at 440 mμ, with two activation maxima at 310 mμ and 360 mμ. The tryptophan content of the effluent was determined from the fluorescence intensity reading at 360 mμ (activation): 440 mμ (fluorescence) by comparison with the intensity reading of different amounts of tryptophan (50–500 ng) processed according to the norharman procedure. The mean recovery of 10 μg tryptophan added in brain homogenates and carried through the procedure was 71 ± 7 (n = 6).

b. Determination of 5-hydroxyindole acetic acid (5HIAA)

5-HIAA was determined by a modification of the spectrophotofluorimetric method of Udenfriend et al. (258). 3–4 ml of the effluent were acidified (pH 1–2) with 100 μl of concentrated HCl, containing ascorbic acid (about 3 $mg/_{ml}$), followed by the addition of KCl to slight saturation, and 4 ml of ethyl acetate before mixing vigorously. After centrifugation at 3,000 xg for 10 min at 4°C, 3.5 ml of ethyl acetate were removed, mixed with 2 ml of 0.5M sodium phosphate buffer pH 7.3 and after centrifugation at 3,000 xg for 10 min at 4°C, the ethyl acetate layer was discarded by aspiration. 0.5 ml of concentrated HCl (containing ascorbic acid, 3 $mg/_{ml}$) was then added and the fluorescence intensity measured with a Perkin-Elmer MPF3 spectrophotofluorimeter at activation 295 mμ and fluorescence wavelength 550 mμ.

The mean recovery of standard 5-HIAA added in brain homogenates and carried through the procedure, compared to pure solutions of standards mixed with 0.5 ml of concentrated HCl (plus ascorbic acid), in respect of their fluorescence intensity, was 74 ± 4 (n = 13).

c. Determination of 3-methoxy-4-hydroxy-phenylethylene glycol (MHPG)

The assay procedure used was that of Walter and Eccleston (259), which, basically involves hydrolysis of MHPG-sulphate complex by using helicase enzyme, extraction of the glycols into ethyl acetate at pH 5, acetylation and extraction of the derivatives formed into dichloromethane and finally, trifluoracetylation and gas-liquid chromatographic determination with a Perkin-Elmer 900 glc fitted with an electron capture detector.

The assay procedure is detailed below: After addition of 0.2 ml of 0.5M sodium acetate buffer (pH 5) to 2-3 ml of the column effluent, helicase was added to give a final concentration of 1.8 $mg/_{ml}$. Finally 2 drops of chloroform were added as anti-bacterial agent, the tubes stoppered, and placed in a water bath at 37°C, and left overnight (17 hours) for hydrolysis of the conjugated MHPG-sulphate.

After the enzymatic hydrolysis, the pH was adjusted to 5.0 with 0.5 ml of 0.5M sodium acetate buffer. Following addition of 4 ml of ethyl acetate, mixing and centrifugation at 3,000 x g for 10 min, 3.5 ml of ethyl acetate were removed and taken into a test tube. The extraction from acetate buffer was repeated and the combined ethyl acetate extracts evaporated to dryness under nitrogen in a heated block at 56°C. The residue was resuspended in 0.4 ml of water and acetylated by the addition of 50 µl acetic anhydride (redistilled) and 0.6 ml $16.5g/_{100ml}$ $KHCO_3$. The solution was gently shaken and the reaction allowed to proceed for 30 min. The sample was then whirlmixed for 1 min with 1.6 ml dichloromethane and centrifuged. 1.3 ml of the lower, organic layer (containing the acetylated derivatives of the glycols) was shaken in a plastic 'Eppendorf' tube

with a little anhydrous Na_2SO_4. The dichloromethane extract was decanted into a glass test tube and evaporated to dryness under nitrogen at 56°C. The residue was resuspended in 0.6ml trifluoracetic anhydride mixture (1 part trifluoracetic anhydride plus 5 parts ethyl acetate, prepared fresh) and the test tube stoppered and heated at 56°C for 15 min. The solution was then evaporated to dryness under nitrogen at 56°C and the residue was taken up in 0.2 ml of re-distilled ethyl acetate, containing 15 $ng/_{ml}$ benzene hexachloride as internal standard. The solution was transferred into a microvial for injection into the glc.

Conditions for the gas-liquid chromatography

A Perkin-Elmer 900 gas-liquid chromatograph was used, fitted with a Perkin-Elmer column (6ft. long, 4mm diameter) which was packed with stationary phase 2.5% silicone gum rubber E301 on a support of chromosorb GAW-DMCS, 80-100 mesh. The carrier gas was argon-methane (90 ± 10%) set at flow rate 60 ml/min (pressure 70 p.s.i.) The column temperature was 165°C, the injection port 220-260°C, the Electron Capture Detector temperature 290-315°C.

4µl of sample were injected each time, with an automatic injection device and the retention time was 1.8 min for MHPG and 2.8 min for the internal standard. The ratio of the height of each MHPG peak to the peak height of the internal standard was calculated and the amount of MHPG in the sample was determined by comparing the sample ratio with the ratio obtained from amounts of MHPG taken through the procedure.

d. Determination of HVA and DOPAC

HVA and DOPAC were determined in 2-3 ml of the effluent which was acidified (pH 1-2) and the procedure continued according to the

method of Pearson and Sharman (41), described in the Appendix.

e. Determination of 5-hydroxytryptamine

5-HT was determined in 2 ml of the column eluate (see flow diagram in Fig. 4.3). After addition of 0.5 ml conc. HCl (containing 3 $mg/_{ml}$ ascorbic acid), the fluorescence intensity of the sample was measured at 300 mμ activation and 530 mμ fluorescence wavelengths, using a Perkin Elmer MPF3 spectrophotofluorimeter.

Standard amounts of 5-HT in 2M H_2SO_4 (the solvent used to elute the amine from the cation exchange resin) were mixed with 0.5 ml conc. HCl (containing ascorbic acid) and the fluorescence intensity was read immediately. The standard curve (arbitrary units of intensity versus ng of 5-HT) served to calculate the amount of 5-HT in the sample solution, which was subsequently corrected for dilution factors, to give the concentration in brain tissue. Recoveries of 5-HT added in brain homogenates and carried through the entire procedure were 87 ± 7 (n = 13).

f. Determination of tryptamine

Attempts were made to measure tryptamine in 3 ml of the column eluate (i.e. $3/5$ of the eluate). The norharman procedure, applied directly on this solution gave a product which had the fluorescence characteristics of the norharman derivative (fluorescence at 440 mμ, activation at 310 and 360 mμ). But after extraction of the eluate into butanol (4 ml) and back extraction into aqueous solution, followed by the norharman procedure, no derivative was formed, which suggests that the fluorescence observed in the first place was due to tryptophan, as it was found by Eccleston et al.(246).

The recovery of standards added in brain homogenates and carried through the procedure was 82 ± 9 (n = 5).

The finding that the tryptamine content of the rat brain does not exceed the limits of sensitivity of the above procedure (about 50 ng) is in agreement with studies of other workers who measured tryptamine in rat brain using various methods and found it to be at the level of 20-40 ng/g tissue (260,261).

g. Determination of normetanephrine

Normetanephrine was measured in whole brain by the radio-enzymatic method of Molinoff et al.(262). The assay depends on the conversion of normetanephrine to the N-methyl derivative of phenylethanolamine N-methyl-transferase (PNMT) with ^{14}C-S-adenosylmethionine (^{14}C-SAM) as a methyl donor. The radioactive product is separated from the ^{14}C-SAM and the other N-methylated amines (such as NA and DA) by the use of alumina resin and by solvent extraction into a mixture of toluene and isoamyl alcohol. The assay procedure is outlined below: The animals were killed by decapitation and the brains removed and immediately frozen on dry ice. Brains were homogenised in 20 µl/mg tissue of 0.4M perchloric acid, ice-cold, and the homogenates centrifuged at 10,000 g for 20 min. To the clear supernatant, ¼ volume of 4M K_2CO_3 was added, left at 0°C for 30 min and after centrifugation at 32,000 g for 15 min at 2-4°C the protein-free supernatant was decanted into a clean test tube. The volume was made up to 2.5 ml with 5mM of ice-cold tris-(hydroxy-methyl)aminomethane (Tris) buffer, pH 8.6, and 400 mg of neutral alumina resin was added. The mixture was left at room temperature for 30 min with occasional mixing, centrifuged at 2,000 g for 15 min. The alumina sediment was kept for the assay of the absorbed NA and

DA, whereas the supernatant contained mainly normetanephrine and 3-methoxytyramine.

25 µl of this supernatant were placed into a 15 ml centrifuge tube and the enzyme assay initiated by the addition of 10 µl of 10 n moles ^{14}C-SAM, 25 µl of partially purified cow adrenal PNMT and phosphate buffer pH 7.9 (50 µM) to make a final volume of 300 µl. The mixture was incubated for 1 hour at 37°C and the reaction was then terminated by the addition of 500 µl of 0.5M borate buffer, pH 10. The radioactive N-methyl-metanephrine was extracted into 6 ml of a mixture of toluene-isoamyl alcohol (3:2) by mixing for 5 min, and centrifugation at 2,000 *g* for 15 min. 4 ml of the toluene-isoamyl alcohol mixture were transferred into a scintillation vial, followed by 1 ml of ethanol and 10 ml of toluene scintillation mixture (containing PPO and POPOP, as described for the GAD assay, 2.2.2c). The radioactivity of samples was determined by scintillation spectroscopy at an efficiency for ^{14}C of about 60%.

Known amounts of normetanephrine were added to homogenates and carried through the procedure as internal standards.

4.2.6 Statistical analysis

As in previous Sections, Student's t test (two tailed) was used for the determination of statistical significance of differences between the mean values obtained from separate groups of animals. The confidence limit used in assessing statistical significance was that the probability (p) value was less than or equal to 0.05.

4.3 RESULTS

4.3.1 Effects of acute administration of L-tryptophan and L-tryptophan plus phenelzine

As shown in Tables 4.1 and 4.2, administration of a single dose of phenelzine (20 $mg/_{kg}$ i.p.), 2 hours before decapitation, was effective in significantly increasing the concentration of 5-HT and decreasing the concentrations of 5-HIAA, HVA DOPAC and MHPG; this treatment had no significant effect on brain tryptophan levels.

Administration of a single dose of L-tryptophan (100 $mg/_{kg}$ i.p.), 1 hour before decapitation, produced a profound increase in brain tryptophan concentration of more than 250 per cent, a significant increase in 5-HT (Table 4.1) and significant increases in 5-HIAA. HVA ($p < 0.05$), DOPAC ($p < 0.025$) and MHPG ($p < 0.05$) (Table 4.2).

When a group of animals were pretreated with phenelzine (20 $mg/_{kg}$ i.p.) 1 hour before injection of L-tryptophan (100 $mg/_{kg}$ i.p.) and 2 hours before sacrifice, brain 5-HT was significantly higher than the concentration of either saline-treated control animals or animals subjected to a single dose of either phenelzine ($p < 0.025$) or L-tryptophan ($p < 0.025$). Tryptophan concentration in brain was not different than after administration of L-tryptophan alone (Table 4.1). After this drug combination, HVA and DOPAC concentrations were significantly lower than control but still significantly higher than after phenelzine alone ($p < 0.05$ and $p < 0.025$, respectively). MHPG was still lower than control ($p < 0.0025$) but not different than after

Table 4.1

Effect of acute or chronic MAO inhibition combined to tryptophan loading on the concentration of 5-HT and tryptophan in the rat whole brain

	Acute treatment		Chronic treatment	
	5-HT ng/g	Tryptophan µg/g	5-HT ng/g	Tryptophan µg/g
Control	369 ± 74(14)	642 ± 0.87(16)	325 ± 37(5)	6.47 ± 0.27(5)
Phenelzine	674 ± 117(6) **	6.87 ± 0.43(6)	805 ± 50(5)**	7.87 ± 0.40(5) **
L-Tryptophan	585 ± 118(6) **	22.27 ± 1.27(6) †	465 ± 26(5)*	21.80 ± 1.46(5) †
L-Tryptophan plus Phenelzine	1064 ± 316(6) ** a,c	21.80 ± 1.95(6) † b	1100 ± 70(5) † b,d	26.13 ± 3.03(5) ** b,c

Acute phenelzine (20 mg/kg i.p.) was given 1 hour before L-tryptophan (100 mg/kg i.p.) and the rats were killed 1 hour later.

Chronic phenelzine (10 mg/kg i.p.) was administered daily, for 15 days, followed 1 hour after the final injection, by L-tryptophan (100 mg/kg i.p.); the rats were killed 1 hour later. Saline solution was injected to the appropriate controls.

Results represent means ± s.d.; the number of animals is in parentheses.

Statistical significance (Student's t test, two-tailed): * $p<0.05$; ** $p<0.025$; ** $p<0.01$; ** $p<0.005$; † $p<0.0005$, compared to control.

a $p<0.025$; b $p<0.005$ compared to phenelzine alone.
c $p<0.025$; d $p<0.0005$ compared to L-tryptophan alone.

Table 4.2

Effect of acute MAO inhibition, alone or in combination with L-tryptophan loading on the amine metabolites in the rat whole brain

Treatment	5HIAA	HVA	DOPAC	MHPG
Control	210 ± 15(6)	144 ± 23(13)	165 ± 15(12)	69 ± 11(5)
Phenelzine (20 mg/kg)	80 ± 19(6) †	74 ± 8(6) †	80 ± 11(6) †	21 ± 2(5) ‡‡
L-Tryptophan (100 mg/kg)	373 ± 54(6) ‡‡	178 ± 31(7) *	191 ± 21(7) **	94 ± 18(7) *
Phenelzine (20 mg/kg) plus L-tryptophan (100 mg/kg)	128 ± 22(6) † c	99 ± 19(5) ** a	109 ± 11(5) ‡‡ b	20 ± 3(5) ‡‡

Amine metabolite concentrations in rat whole brain following a single administration of phenelzine and L-tryptophan.

The animals were first given phenelzine, followed 1 hour later by L-tryptophan and were sacrificed 1 hour after the latter injection.

Saline injections were given instead of one of the two drugs when the other had to be administered alone.

Results (μg/g tissue) represent means ± s.d.; number of animals is in parentheses.

Statistical significance (student's t test, two tailed): * $p<0.05$; ** $p<0.025$; ‡ $p<0.01$; ‡‡ $p<0.0025$; † $p<0.0005$ compared to control

a $p<0.05$; b $p<0.025$; c $p<0.01$ compared to single phenelzine

phenelzine alone. Similarly, 5-HIAA was significantly lower than control but still higher than after phenelzine alone ($p < 0.01$) (Table 4.2).

Paradoxically, normetanephrine, the amine metabolite of noradrenaline did not rise after acute administration of phenelzine or phenelzine plus L-tryptophan, although L-tryptophan alone increased significantly the concentration of this amine ($p < 0.025$). Pargyline (50 $mg/_{kg}$ i.p.) (a MAO inhibitor) on the other hand, after acute administration, did give a significant rise in normetanephrine ($p < 0.025$) (Table 4.4).

4.3.2 Effects of chronic administration of phenelzine

The results obtained after administration of phenelzine for 15 days are detailed in Tables 4.1 and 4.3.

Treatment of rats with phenelzine (10 $mg/_{kg}$ i.p. daily) produced highly significant increases in 5-HT and in tryptophan (Table 4.1) and significant reductions in 5-HIAA, HVA, DOPAC and MHPG (Table 4.3). L-tryptophan (100 $mg/_{kg}$ i.p.), given acutely 1 hour after the last (15th) injection of phenelzine gave results comparable to its administration after acute phenelzine, that is significant increases in tryptophan and 5-HT and significant decreases in 5-HIAA, HVA, DOPAC and MHPG, compared to saline treated control animals.

Compared to chronic phenelzine treatment, without L-tryptophan administration after the 15th injection, the combination of the two drugs produced highly significant increases in 5-HT and tryptophan (Table 4.1) and significant increases in the concentrations of the metabolites, 5-HIAA ($p < 0.025$), HVA ($p < 0.05$), DOPAC ($p < 0.0025$) and MHPG ($p < 0.05$) (Table 4.3). At this point it should be noted that

Table 4.3

Effect of chronic MAO inhibition, alone or in combination with L-tryptophan loading, on the amine metabolites in the rat whole brain

Treatment	5-HIAA	HVA	DOPAC	MHPG
Control	218 ± 13(5)	141 ± 21(6)	176 ± 14(6)	70 ± 14(5)
Chronic phenelzine	59 ± 6(5)	68 ± 13(5)***	62 ± 12(5)$^{\mp}$	12 ± 1(5)$^{\mp}$
Acute L-tryptophan	469 ± 42(5)**	179 ± 17(5)**	225 ± 19(5)***	100 ± 7(5)**
Chronic phenelzine plus Acute L-tryptophan	83 ± 9(5)$^{\mp}$b	98 ± 13(5)***a	117 ± 20(5)***c	16 ± 2(5)$^{\mp}$a

Phenelzine (10 mg/kg i.p.) was administered daily, for 15 days, followed, 1 hour after the final injection, by L-tryptophan (100 mg/kg i.p.); the rats were sacrificed 1 hour later.

Saline solution was injected to the appropriate controls.

Results/$\mu g/g$ represent means ± s.d.; number of animals is in parentheses.

Statistical significance: *$p < 0.05$; **$p < 0.025$; ***$p < 0.01$; $^{\mp}p < 0.0025$, compared to controls
a $p < 0.05$; b $p < 0.025$; c $p < 0.0025$, compared to chronic phenelzine alone.

Table 4.4

Effect of acute or chronic MAO inhibition on the concentration of normetanephrine in the rat whole brain.

Treatment	Normetanephrine ng/g tissue
Control (saline)	50.4 ± 8.9(13)
Acute phenelzine (20 mg/kg i.p.)	49.7 ± 10.6(5)
Acute phenelzine (20 mg/kg i.p.) plus L-tryptophan (100 mg/kg i.p.)	41.9 ± 8.4(6)
L-tryptophan (100 mg/kg i.p.)	69.9 ± 7.1(5) *
Acute pargyline (50 mg/kg i.p.)	62.8 ± 6.0(8) *
Chronic phenelzine (10 mg/kg i.p., 15 days)	63.5 ± 3.2(8) **

Normetanephrine was measured as described in the text (4.2.5 g), following acute or chronic (for 15 days) administration of phenelzine, acute administration of pargyline or L-tryptophan and acute administration of the combination phenelzine plus L-tryptophan. Control rats were injected saline solution.

The animals were killed 2 hours after acute phenelzine or pargyline, 1 hour after acute L-tryptophan and 2 hours after the last of 15 daily injections of phenelzine.

Values represent means ± s.d., number of animals is in parentheses.

Statistical significance (Student's t test, two tailed):

* $p<0.025$; ** $p<0.005$ compared to control

MHPG had not risen after acute administration of phenelzine plus L-tryptophan compared to acute phenelzine administration (Table 4.2). Chronic treatment of rats with phenelzine (10 mg/kg i.p., for 15 days), in contrast to a single injection of this MAO inhibitor, produced a significant elevation of normetanephrine ($p < 0.005$) (Table 4.4).

4.3.3 Effect of L-tryptophan on DA and NA concentrations

A single administration of L-tryptophan (100 mg/kg i.p.), 1 hour before sacrifice, produced a significant increase in brain DA concentration ($p < 0.025$) but had no significant effect on the concentration of NA (Table 4.5).

4.3.4 Effect of L-tryptophan on HVA and DOPAC in various brain areas

As shown in Table 4.6, administration of a single dose of L-tryptophan (100 mg/kg i.p.), 1 hour before sacrifice, produced significant increases in both HVA and DOPAC in the corpus striatum ($p < 0.005$ and $p < 0.025$, respectively), the nucleus accumbens ($p < 0.025$) and the olfactory tubercle ($p < 0.05$ and $p < 0.005$, respectively) as has been found in the rat whole brain (Table 4.2).

4.3.5 Effect of DL-amphetamine administration on 5-HT, DA and NA metabolism

A high dose of DL-amphetamine (16 mg/kg i.p.), 1 hour before sacrifice, produced no significant effect on 5-HT concentration in the whole brain, but significant increases in tryptophan ($p < 0.025$), 5-HIAA ($p < 0.01$) and MHPG ($p < 0.005$). On the other hand, DOPAC concentration was significantly lowered ($p < 0.0005$) but HVA concentration was significantly increased ($p < 0.05$) (Table 4.7).

Table 4.5

Effect of L-tryptophan on rat whole brain concentrations of dopamine and noradrenaline

Treatment	Dopamine ng/g tissue	Noradrenaline ng/g tissue
Control	1380 ± 44(5)	651 ± 52(5)
L-tryptophan	1690 ± 205(5) *	700 ± 75(5)

L-tryptophan (100 mg/kg i.p.) was administered 1 hour before sacrifice.

Control animals received o.5 ml of saline solution.

Values represent means ± s.d.; number of animals is in parentheses.

* $p < 0.025$ compared to control values (Student's t test)

Table 4.6

Effects of L-tryptophan loading of rats on regional DA metabolism

	Control (saline)		L-Tryptophan	
Brain Area	HVA	DOPAC	HVA	DOPAC
Corpus striatum	1.13 ± 0.22(6)	1.11 ± 0.10(6)	† 1.76 ± 0.13(6)	** 1.63 ± 0.32(6)
Nucleus accumbens	1.47 ± 0.33(7)	1.78 ± 0.22(7)	** 2.04 ± 0.34(6)	** 2.20 ± 0.32(6)
Olfactory tubercle	1.53 ± 0.29(7)	1.48 ± 0.20(6)	* 1.89 ± 0.24(7)	† 2.26 ± 0.42(7)

L-tryptophan (100 mg/kg i.p.) was administered 1 hour before sacrifice and the concentrations of HVA and DOPAC in regions of rat brain were measured.

Control animals were treated with saline solution.

Values represent means ± s.d.; number of animals is in parentheses.

Statistical significance (Student's t test; two-tailed):

* $p<0.05$; ** $p<0.025$; † $p<0.005$, compared to control.

Table 4.7

Effect of DL-amphetamine on rat whole brain concentrations of 5-HT 5-HIAA, tryptophan, HVA, DOPAC and MHPG

	Control	DL-Amphetamine	Statistical significance (Student's t test)
5-HT ng/g tissue	323 ± 72(8)	395 ± 83(8)	n.s.
5-HIAA ng/g tissue	192 ± 30(8)	254 ± 41(8)	$p<0.01$
Tryptophan µg/g tissue	5.98 ± 1.18(8)	7.96 ± 1.04(8)	$p<0.025$
HVA ng/g tissue	143 ± 16(9)	158 ± 14(9)	$p<0.05$
DOPAC ng/g tissue	163 ± 15(7)	76 ± 18(8)	$p<0.0005$
MHPG ng/g tissue	70 ± 11(7)	92 ± 11(8)	$p<0.005$

A dose of 16 mg/kg i.p. of the drug was given to rats 1 hour before sacrifice; saline solution was injected to animals that served as controls.

Values represent means ± s.d.; the number of animals is in parentheses.

4.3.5 Behavioural effects of L-tryptophan plus phenelzine

In addition to the behavioural effects described in Section 5 (5.3.4c), of which hyperactivity is the most obvious, this drug combination induced in rats with a unilateral 6-OH-DA lesion of the nigrostriatal DAergic pathway (performed as described in Section 2) circling behaviour ipsilateral to the lesioned side. The turning was slow (about 2 turns/min) and persisted for about 1 hour. It was only observed after the drug combination and not after any one of the two drugs alone. The direction of turning was the same as with amphetamine, i.e. ipsilateral to the lesion (8,110).

4.4 DISCUSSION

4.4.1 Effects of MAO inhibition plus L-tryptophan loading on monoamine metabolism

Earlier biochemical studies showed that treatment of animals with a MAO inhibitor such as phenelzine, resulted in an apparent decrease in the concentrations of the deaminated metabolites of 5-HT, DA and NA and an increase in the concentrations of the parent amines in the brain (264,237). Acute treatment with L-tryptophan, on the other hand resulted in a marked increase in the concentrations of tryptophan, 5-HT and 5-HIAA in the rat brain (220). The data from this study are in agreement with these findings and provide further evidence for the predicted effects of the drugs. Thus, single administration of the MAO inhibitor phenelzine reduced significantly the concentrations of 5-HIAA, HVA, DOPAC and MHPG. A single administration of L-tryptophan (100 $mg/_{kg}$ i.p.) increased the concentrations of tryptophan, 5-HT and 5-HIAA in brain.

Combination of phenelzine with the precurser of 5-HT tryptophan resulted in an increased synthesis and turnover of 5-HT compared to the administration of the inhibitor alone. Despite the inhibition of MAO, loading with L-tryptophan resulted in an increase in 5-HIAA (compared to MAO inhibition alone) and in 5-HT one hour after the injection. The increased rate of synthesis of 5-HT after acute or chronic treatment with phenelzine (Table 4.1) may be a result of an increased concentration of total tryptophan or an altered

compartmentalisation of tryptophan in brain. An increased availability of tryptophan may lead to an increased synthesis and turnover of the functional pool of 5-HT, since tryptophan hydroxylase is not saturated at normal physiological levels of tryptophan. These results of L-tryptophan loading on 5-HT parameters again confirm findings of similar studies (264) and suggest that the treatment with the precursor was effective in increasing the synthesis and turnover of 5-HT, regardless of whether MAO was inhibited or not. The reason for this effect of L-tryptophan on rats treated with a MAO inhibitor could be either the existence of two or more forms of MAO (265), one of which may not be inhibited by phenelzine, or the non complete inhibition of MAO at this dosage of phenelzine (20 $mg/_{kg}$ i.p.). Phenelzine, however, is probably a non-selective MAO inhibitor (263) and is effective in blocking the MAOs responsible for oxidising both 5-HT and catecholamines. The concentrations of the amine metabolites were reduced to approximately the same extent, but not completely. Our finding (Nicolaou, unpublished) that a higher dose of phenelzine (30 $mg/_{kg}$ i.p.) caused even higher percentage reductions of the metabolites, indicates that MAO was not completely inhibited and, therefore, the increased availability of the substrate (5-HT) activated the enzyme to produce the observed increase of 5-HIAA and HVA, DOPAC.

In addition to the effects on 5-HT synthesis and metabolism, tryptophan loading, with or without MAO inhibition, produced effects on synthesis and metabolism of other monoamines, such as DA and NA. L-tryptophan alone produced a significant increase in HVA and DOPAC concentrations in the striatum and the limbic system, which was probably reflected in an elevation of the concentration of DA and its

major metabolites in the rat whole brain. Similarly, the concentration of total MHPG, the major brain metabolite of NA (228), was increased significantly following tryptophan administration; the concentration of normetanephrine, the intermediate amine metabolite of NA, was also increased, but there was no effect on NA concentration.

The increase in catecholamine metabolite concentrations could be a result of an increased release, synthesis, turnover or of impairment of transport of the metabolites out of the brain due to competition with the abundantly formed 5-HIAA.

In all probability, the brain monoamines as well as normetanephrine, cannot be transported out of the brain; The finding, therefore, that normetanephrine is increased after L-tryptophan, in parallel with the acid metabolites, seriously weakens the possibility that competition for a transport mechanism is responsible for the increase of the deaminated metabolites in brain. The finding that administration of L-tryptophan to dogs pretreated with phenelzine caused a significant increase in the concentration of HVA in the CSF (251,264) also argues against this possibility. The active transport system which is sensitive to probenecid, removes the acid metabolic products of the amines from the brain to plasma (266,38) and, the suggestion that tryptophan loading may block this system, could be considered. However, although most of the HVA formed in brain is transferred by this mechanism, DOPAC is only partially transported by the probenecid-sensitive active transport system (42). Therefore, the almost equal degree of increase in the concentrations of HVA and DOPAC after L-tryptophan administration eliminates the possibility of involvement of this mechanism in the observed changes in DA metabolism. For the same reason, the possibility that the increase in HVA and DOPAC is caused by interference with the DA reuptake into

its nerve terminals by tryptophan, (or 5-HTP or 5-HT) seems unlikely, considering the proposed concept of DOPAC formed intraneuronally and HVA formed extraneuronally (38,39,40).

A simple, reserpine-like increase in the release of DA and NA, probably due to displacement from their neurons by newly synthesised 5-HT should result in a reduction in the amine levels of the brain. The lack of effect on NA and the increase in DA (shown in Table 4.5) do not support this very simple interpretation. On the other hand, the increased synthesis or turnover of DA and NA, suggested by these data could be a secondary result of displacement of these amines from granular storage sites by the 5-HT which 'spills over' the neurons.

An acute injection of the non-selective MAO inhibitor pargyline (265) produced a rise in normetanephrine, as well as a fall in MHPG, whereas the other MAO inhibitor, phenelzine, caused a similar change only after it was administered chronically. The difference between the two drugs could be due to a different degree of MAO inhibition or to the possibility that pargyline is a specific inhibitor of the MAO which is metabolising normetanephrine. However, this possibility seems unlikely, since both drugs are known to be non-selective MAO inhibitors (263,265). Therefore, the difference may be due to the different degree of MAO inhibition they produced, which is known to be dose-dependent (242).

Chronic administration of the MAO inhibitor phenelzine did not alter the profile of the acute effects of this drug. A relatively higher degree of inhibition is indicated by the lower acid metabolite levels. However, tryptophan loading of rats, after acute or chronic administration of phenelzine, was capable of increasing significantly the concentrations of the metabolites (5-HIAA, HVA, DOPAC, MHPG). An

increase of the catecholamine synthesis or turnover seems to be suggested by these results, rather than an effect on the probenecid-sensitive active transport system.

4.4.2 Release of catecholamines by 5-hydroxytryptamine

The release process is more probably the system affected by L-tryptophan loading. The neuroanatomical background is available for such interpretation. Following the administration of 5-HTP to rats, the accumulation of 5-HT in catecholaminergic neurons has been demonstrated using histofluorescence techniques (267,248,268), presumably arising through the decarboxylation of 5-HTP in the catecholaminergic neurons. In vitro studies by various investigators presented evidence supporting the above in vivo results. Ng et al. (269) showed that both 5-HT and the precursor 5-HTP at concentrations 10^{-5}M are able to increase the efflux of labelled DA from striatal slices. In this system, the decarboxylation of 5-HTP was found to be necessary for the 5-HTP-induced efflux of DA, a point implicating 5-HT as the stimulatory agent for this effect of 5-HTP. Furthermore, it was found by other workers that both 5-HT and 5-HTP can increase the efflux of endogenous DA from rat striatal synaptosomes (270). These in vitro systems, i.e. slices and synaptosomes, are believed to be devoid of intact interneuronal or transynaptic processes; this makes the possibility of an indirect effect of increased 5-HT neuronal activity onto catecholaminergic neurons with subsequent changes in the metabolism of DA or NA, as a very unlikely interpretation of the findings of the present study. Thus, it seems likely that increases in 5-HT may produce alterations in catecholamine functioning, by way of effects within the catecholamine neuron.

5-HT has been found to inhibit catecholamine accumulation in storage vesicles from the adrenal medulla (279) and also DA accumulation in storage vesicles from the striatum (280). These findings are consistent with the concept of intraneuronal displacement of catecholamines by 5-HT, but they also imply an inhibition of synthesis of the catecholamines accompanying the stimulatory effect (270). Thus, despite the differences, the findings of the present study are compatible with the idea of intraneuronal displacement of DA and NA from their vesicular storage sites and release into the intra- and extra-neuronal cytoplasm where they are metabolised by the oxidative and methylating enzymes.

However, the inhibition of DA synthesis by 5-HT and 5-HTP in rat striatal synaptosomes in vitro at concentrations 10^{-5}M 5HT (30% inhibition) and 2 x 10^{-4}M 5-HTP (70% inhibition) (270), thought to result from the release of DA and the consequent increase in DA-induced feedback inhibition of the enzyme tyrosine hydroxylase or from a direct inhibition of dopa decarboxylation, does not seem to agree with the finding of the present study, i.e. the increase in DA or no change in NA levels following loading with the precursor of 5-HT. Both 5-HT and 5-HTP were able to release endogenous DA at concentrations 2 x 10^{-4}M (270). The different methodological approach used in these in vitro experiments and the present in vivo study impair any comparison of the results and may account for the apparent discrepancy. However, the uptake system for 5-HT in brain seems to be more complex. Shaskan and Snyder (271) demonstrated that (^{3}H)-5HT can accumulate in catecholamine neurons or in serotonergic neurons depending on the concentration of 5-HT. Thus, striatal slices could take up labelled 5-HT at concentrations 10^{-8}M, but at higher

concentrations a greater proportion of amine entered catecholamine neurons by a low affinity uptake system ('uptake 2'), in contrast to the high affinity uptake into serotonergic neurons ('uptake 1').

Subsequent experiments also support the finding that increased 5-HT caused an increase in catecholamine turnover. The combination of MAO inhibition, either acute or chronic, and tryptophan loading caused an increase in 5-HT concentration in brain, which was the sum of the effects of the two treatments separately (Table 4.1). Thus, MAO inhibition together with tryptophan loading might cause displacement of catecholamines to a greater extent than a single dose of L-tryptophan, if 5-HT induced displacement of catecholamines from the nerve endings is proportional to the concentration of 5-HT. The demonstrated uptake of 5-HT and 5-HTP into catecholamine neurons in the brain, previously depleted of their catecholamines (267,268) indicates that 5-HT (and probably 5-HTP) are located outside the storage granules, possibly fixed to extragranular binding sites and under the attack of intraneuronal MAO. Therefore, the enzyme has to be inhibited in order to obtain an accumulation of 5-HT. However, L-tryptophan alone produced significant increases in the turnover of NA and the synthesis and turnover of DA in the rat whole brain, as it probably did in the corpus striatum and the limbic areas (nucleus accumbens, olfactory tubercle) of the brain with regard to DA.

This may be difficult to reconcile with the reported ineffectiveness of L-tryptophan to produce marked hyperactivity in rats (234) or turning behaviour in rats with nigrostriatal pathway lesions (present study), in contrast to the effectiveness of the combination with MAO inhibition. The DAergic and probably the NAergic component of the effects of this drug combination seem to be present after a simple increase of 5-HT in brain. This may indicate that the low

affinity 'uptake 2' system for the entry of 5-HT into catecholamine neurons proposed by Shaskan and Snyder (271) is possibly functioning in vivo; their finding that as low as 10^{-8}M 5-HT could be taken up by catecholamine neurons may not be incompatible with the lack of obvious behavioural responses of rats at low doses of 5-HT precursors or MAO inhibitors alone (234). A slight amount of released DA or NA may not reach the appropriate receptors mediating their behavioural effects because of degradation by intra- or extraneuronal MAO. The fact that there is no increase of acid metabolites after the drug combination compared to L-tryptophan alone, may be accounted for by the inhibition of their formation by phenelzine. However, there is some evidence of an increased turnover of DA after L-tryptophan plus phenelzine, in that the reduction in the concentrations of HVA and DOPAC after phenelzine alone was significantly diminished when phenelzine was given together with L-tryptophan (Table 4.2).

4.4.3 Amphetamine-like action of L-tryptophan loading

Amphetamine and the combination of L-tryptophan with a MAO inhibitor are known to present a common feature behaviourally, in that they induce a hyperactivity syndrome in rats (272,234). Amphetamine is known to influence all three monoamine systems, i.e. DAergic, NAergic and serotonergic (273, and Table 4.7 of the present study). It has also been demonstrated that the monoamine releasing properties of amphetamine at low concentrations (10^{-7} - 10^{-4}M) in vitro (242) or at low doses after intraperitoneal administration (43,275) are accompanied at higher concentrations (10^{-3}M) by amine reuptake inhibition and MAO inhibition (242). This complex effect of amphetamine was demonstrated in vitro (242) and in vivo (274). The finding of Kuczenski (274), that the effect of amphetamine on

striatal DA dynamics has, at least, two components, one resulting in an increase and the second in a decrease in the conversion of (^{3}H)-tyrosine to (^{3}H)-DA, does not exclude the uptake inhibition as a possible mediator of the observed effects on the levels of the deaminated metabolites. Similar double effects of amphetamine have been reported by Rutledge et al.(242) with regard to NA and 5-HT release and reuptake in vitro.

The present study, as it is shown in Table 4.7., confirmed some of the earlier observations: amphetamine is indeed affecting the DA, NA and 5-HT systems. Although in these experiments, the effects of DL-amphetamine administered as a single high dose (16 mg/kg i.p.) were determined only in relation to the concentrations of the acid metabolites HVA, DOPAC and MHPG and not of the amine levels in the whole brain, the results could be interpreted in several ways. The observed selective decrease of DOPAC, with an opposite effect on HVA, could be due to DA reuptake inhibition and support the concept of mainly intraneuronal formation of DOPAC and extraneuronal formation of HVA; the same finding was reported by several investigators estimating the same metabolites (22,40,38). The results obtained by them referred to the striatum and the mesolimbic system, but the whole brain picture with regard to DA metabolism should not be different, as these brain structures are the richest in DA and its metabolites.

The finding that a high dose of amphetamine (known to be effective at low doses in releasing DA and consequently increasing HVA,) had significant effect on the whole brain concentration of this metabolite, could be attributed to the MAO inhibition that may occur at this dosage and act as a kind of homeostatic mechanism, to maintain normal DA levels. The possibility that the effect of DL-amphetamine

on DA metabolite concentrations is the result of an influence of this drug on the system responsible for the active transport of deaminated metabolites out of the brain (i.e. the probenecid-sensitivesystem) could not explain these results. An inhibition of this system would be expected to have no effect on DOPAC but to increase the HVA concentration, as does probenecid (38,39); DL-amphetamine, however, caused a significant reduction of DOPAC concentration in brain, in addition to an increase in HVA.

The possibility that transynaptic interactions mediate the effects of the amphetamine treatment on the various monoamine systems would imply that the observed effects on the metabolism of the three monoamines are not all resulting from a direct action; a primary effect on the catecholamines, for example, might mediate some of the effects on the 5-HT system. However, the complexity of these monoamine interactions (as discussed in detail in Section 5) cannot be simplified by whole brain studies, but can only be studied by the use of other more direct approaches and by investigations restricted to specific brain areas.

The increased tryptophan and 5-HIAA with unchanged 5-HT concentrations in brain after DL-amphetamine (shown in Table 4.7) are indicative of an increase in the synthesis and turnover of 5-HT. The increase in MHPG suggests that the turnover of NA is also increased, but unlike DA, blockade of NA reuptake does not seem to be a significant component of the action of DL-amphetamine on this amine (at this dosage). Such data seem to indicate that monoamine release and inhibition of reuptake can lead to increased turnover, probably due to homeostatic control of neuronal activity, mediated by unknown feedback mechanisms.

The biochemical response to DL-amphetamine can be summarised as being constituted by an increased synthesis and metabolism of 5-HT, following the stimulation of its release or the inhibition of its reuptake and perhaps MAO inhibition, in addition to the stimulation of release of DA from its neurons and probably the release and increased metabolism and/or turnover of NA. The changes in these processes seem to present, in general, a close correlation with the corresponding changes in the three monoamine systems following chronic or acute administration of phenelzine together with L-tryptophan.

The observed in the present study similarity between the behavioural responses to DL-amphetamine and to L-tryptophan plus phenelzine, i.e. hyperactivity and ipsilateral turning of rats with a unilateral lesion of the nigrostriatal DAergic pathway, seem to support the hypothesis that these treatments have a similar mode of action.

The finding that the hyperactivity syndrome in the rat, produced by MAO inhibition combined to L-tryptophan could be abolished by pretreatment of the animal with α-methyl-p-tyrosine (an inhibitor of catecholamine synthesis) or chlorpromazine (a DA receptor blocker) but was not affected by NA depletion with disulfiram (a D-b-h inhibitor) (249,250), suggests that brain DA rather than NA has an important role in the behavioural expression of the accumulation of 5-HT in brain. Since pretreatment with α-methyl-p-tyrosine did not affect 5-HT synthesis, but inhibited the responses to the 5-HT receptor agonist 5-methoxy-N,N-dimethyltryptamine (5-MDT), it appears that DAergic neurons postsynaptic to the serotonergic neurons mediate certain behavioural effects of 5-HT receptor stimulation (250).

Thus, it seems that the effect on the DAergic system may be an essential component of the behavioural response to the treatment with a MAO inhibitor plus L-tryptophan. The involvement of the NAergic system, although less clear, may be of minor importance.

Administration of L-dopa (the precursor of DA and NA) to rats pretreated with a MAO inhibitor also resulted in hyperactivity (256), thought to be partly due to displaced 5HT from serotonergic neurons (245,256) and partly due to increased DA in brain (276). Although both 5-HT and DA appear to be involved in the production of the behavioural syndrome of hyperactivity, transynaptic contacts of the respective neurons seem to mediate this response, with the DAergic neurons in the dominant role (250,252).

Several questions related to the amphetamine-like effects of the combination of phenelzine with L-tryptophan remain unanswered. Firstly, it is not clear whether L-tryptophan is taken up by catecholamine-containing neurons and subsequently converted to 5-HTP and finally 5-HT, which acts to release DA or NA; or, whether the conversion to 5-HT takes place in serotonergic neurons and then released 5-HT enters catecholamine neurons to cause the displacement of DA and NA from their nerve terminals; or, finally, whether L-tryptophan is converted to 5-HTP in serotonergic neurons and then 5-HTP is taken up by catecholamine neurons and decarboxylated to form 5-HT, which releases DA or NA. Secondly, it is possible that an intermediate product of the conversion of L-tryptophan to 5-HT or tryptamine or a 5-HT derivative, could act as a false transmitter to cause the release of DA and NA from their neurons. The presence of tryptamine in reserpine-resistant storage sites within catecholamine neurons was suggested by the work of Marsden and Curzon (260).

The possibility that this amine contributes to the biochemical and behavioural effects of the combination phenelzine plus L-tryptophan, either by catecholamine release or by interaction with 5-HT receptors, cannot be discounted, although 5-HT rather than tryptamine appears to be responsible for the hyperactivity syndrome (234).

Several experimental and clinical data indicate that an increase of 5-HT at the functional sites of the brain may not be the total explanation for the effectiveness of the combination tryptophan plus phenelzine in some patients suffering from affective disorders. From the studies detailed here, it seems possible that this drug combination may act by increasing the activity of other than the 5-HT monoaminergic systems in a manner similar to amphetamine. Administration of high doses of L-tryptophan or 5-hydroxytryptophan has been used in an attempt to alter the functioning of serotonergic neurons in the brain of psychiatric or neurological patients (254). Failure to improve the depressive state may be due to the fact that a simple deficiency of brain 5-HT does not seem to be the only reason for the syndrome; precursor loading alone demonstrated little beneficial effect in depression (277), but the ability of L-tryptophan to potentiate MAO inhibitors in the treatment is widely accepted (278).

The involvement of the catecholamines in the pharmacological profile of this treatment may be another important point for the interpretation of its therapeutic effectiveness, the elucidation of the mechanism of action and the search for improved effective treatments for affective disorders. Drugs like amphetamine, appearing to have a similar pharmacological profile, could form the basis for the replacement of this treatment. They could also clarify the mechanism of the various forms of depressive illness by comparison of

the biochemical and behavioural effects in laboratory animals to the clinical symptoms in humans.

4.4.4 Conclusions

1. Treatment of rats with L-tryptophan, with or without a MAO inhibitor, produced an increase in the synthesis and turnover of 5-HT in brain, but also in the synthesis and/or turnover of DA and NA. Among the various possible mechanisms, displacement of the catecholamines from their storage granules by 5-HT or 5-HTP or even by tryptamine that 'spills over' the catecholamine neurons, appears to predominate.

2. The combination of MAO inhibition with tryptophan loading seems to have similar but different effects on the three monoamines (5-HT, NA, DA) in brain. The similarities, and especially the monoamine-releasing properties, lead to a possible interpretation of the effectiveness of the combination MAO inhibitor plus L-tryptophan in some cases of depression in humans and may indicate ways for improved treatments of this syndrome.

3. Although the accumulation of 5-HT in brain and the consequent effects on catecholamine metabolism present a rather unphysiological situation, the amphetamine-like effects of increased 5-HT synthesis and turnover on catecholamine function may occur in addition to the intraneuronal, receptor mediated interaction of 5-HT and catecholamines. The influence of 5-HT on DAergic neuronal activity, in particular, is examined in Section 5.

SECTION 5

SOME BIOCHEMICAL AND BEHAVIOURAL OBSERVATIONS OF RATS WITH ASYMMETRIC ELECTROLYTIC LESIONS IN THE MEDIAN RAPHE (MR) OR IN THE DORSAL RAPHE (DR) NUCLEI

5.1 INTRODUCTION

5.1.1 5-Hydroxytryptamine in brain

The presence of 5-hydroxytryptamine or serotonin (5-HT) in the rat brain has been convincingly demonstrated by the use of biological and chemical methods (282,281,247). It was found to be unevenly distributed, with the highest concentrations being present in hypothalamus, brain stem, striatum and some areas of the limbic system (282,281). Further work showed tryptophan hydroxylase, the rate-limiting step in 5-HT biosynthesis, to be present in the brain (216,217) with its regional activity correlating with the distribution of serotonergic nerve cells and their processes (218). Evidence that 5-HT in brain fulfills the role of a neurotransmitter was obtained from physiological (29,283), pharmacological (284) and histochemical (5) studies. All these observations point to the existence of specific monoaminergic neurons utilising 5-HT, which is stored in vesicles and can be released from the nerve terminals by the transmission of nerve impulses (286,283) to act on specific receptors (287).

The corpus striatum of the rat brain has a relatively high concentration of 5-HT (282,281,288), unequally distributed in various parts of this structure, but concentrated mainly in the ventro-caudal part (289). High activities of tryptophan hydroxylase and L-aromatic aminoacid decarboxylase, the biosynthetic enzymes for 5-HT, were found (282,218). A high affinity uptake and release process for 5-HT has also been demonstrated in striatal slices in

vitro (271). The rat substantia nigra concentration of 5-HT and the activity of tryptophan hydroxylase are among the highest in the brain, almost equally distributed within the areas pars compacta and pars reticulata (282). A high affinity uptake (73) and release (290) process for exogenous 5-HT has been demonstrated.

5.1.2 Location of cell bodies of serotonergic neurons (Fig. 5.1)

Fluorescence histochemical studies have demonstrated that the cell bodies of the 5-HT neurons are mainly localised in the raphe nuclei of the lower brain stem (5,291), named B1 to B9 by Dahlström and Fuxe (5). Cell bodies in the medulla oblongada and the pons (B1 to B6) generally give rise to descending bulbo-spinal pathways (5,291), whereas ascending pathways generally originate in the caudal raphe nuclei of the mesencephalon (5,292), named B7, B8,B9. The cell bodies of group B7 are situated in the substantia grisea centralis. The vast majority of cell bodies lie within the nucleus dorsalis raphe (DR), especially in the part just above and medial to the fasciculus longitudinalis (FL). The cells of group B8 are present from the caudal end of the posterior colliculus to the end of the nucleus interpeduncularis, being situated mainly within the nucleus medianus raphe (MR). Some cells are also present within the caudal portion of the nucleus linearis, and a number of cells are found just lateral to the MR within the formatio reticularis (reticular formation RF). Group B9 is present mainly within and around the lemniscus medialis (LM) from the caudal end of the posterior collicle to the caudal end of the nucleus interpeduncularis. A number of cells are also present dorsal to the lemniscus medialis within the mesencephalic reticular formation. Fig. 5.1 depicts a transverse diagram of the rat midbrain, showing the major raphe cell groups and the adjacent areas.

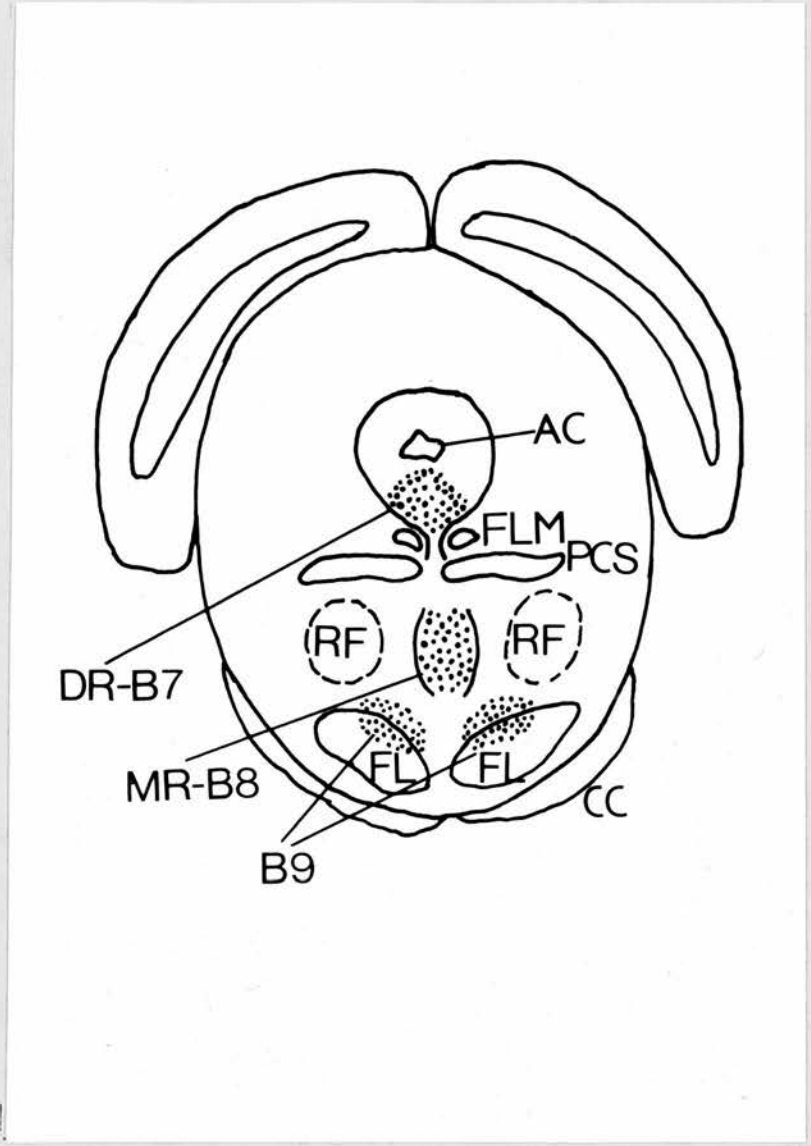


Fig. 5.1

Diagrammatic transverse representation of the midbrain showing the location of the major raphe cell groups (dotted areas) and the adjacent brain areas.

Abbreviations

AC : aqueductus cerebri
DR-B7: dorsal raphe (area B7 of Dahlström and Fuxe,5)
MR-B8: median raphe (area B8)
PCS : pedunculus cerebellaris superior
RF : formatio reticularis (reticular formation)
FLM : fasciculus longitudinalis medialis
CC : crus cerebri
FL : fasciculus longitudinalis

High concentrations of 5-HT have been found in several of the raphe nuclei where 5-HT containing cell bodies are located. The nucleus raphe dorsalis has the highest concentration of 5-HT in the rat brain (282). Tryptophan hydroxylase is especially concentrated in the raphe nuclei, with the highest concentration among all the brain areas being found in the nucleus raphe dorsalis (282).

The 5-HT nerve cells are thus found almost entirely in the raphe nuclei. The importance of this is not clear, since the connections and the functions of these nuclei are more or less unknown. According to Taber et al. (293), the raphe complex seems to be 'a primitive part of the brain which shows relatively little differentiation during the phylogenetic ascent of the vertebrates' and may thus have 'relatively small but fundamental and important tasks in the function of the brain.' Some of the nuclei send descending fibres to the spinal cord. Brodal et al. (294,295) have shown that all the raphe nuclei give rise to ascending fibres and that these fibres appear to be part of links in the ascending pathways from the spinal cord to higher levels.

5.1.3 Serotonergic nerve terminals in brain

5-HT-containing nerve terminals have been demonstrated, mainly by the use of histochemical fluorescence, in the nucleus suprachiasmaticus, in the ventral part of the lateral geniculate body, in the fornix, in the habenula and other thalamic nuclei (6,296), in certain amygdaloid nuclei, in the hippocampus (6), in the globus pallidus (6,296) and in the olfactory tubercle (297). Also, very fine 5-HT nerve terminal plexuses have been visualized in the septal area, in the anterior colliculi of the preoptic area, in the hippocampal formation, in the neo- and mesocortex (6) and in the nucleus caudatus-putamen (corpus striatum) (296,6). A cerebellar sero-

tonergic innervation has also been demonstrated (298) and strong evidence exists that the substantia nigra receives a large number of serotonergic fibres forming axo-dendritic synapses (73,5,297).

The presence of 5-HT binding sites, thought to be associated or to be part of the 5-HT postsynaptic receptor, as well as the presence of a 5-HT-sensitive adenylate cyclase linked to them, have been demonstrated in close correlation with the distribution of serotonergic nerve terminals in various brain areas, e.g. cerebellum, cerebral cortex, striatum, hippocampus, colliculi, hypothalamus (287,299,300).

5.1.4 Raphe-efferent projections

The majority of the ascending serotonergic raphe-efferents sweep ventrally from the raphe nuclei, then curve rostrally to course through the ventral tegmentum and into the medial forebrain bundle (MFB) (7). From the MFB, fibres branch into the hypothalamus, preoptic area, anterior amygdala and olfactory tubercle, while some fibres enter the fornix or the stria terminalis and the hippocampal formation (301). A well defined lateral mesencephalic-cortical serotonergic pathway has been demonstrated, together with a medial subcortical pathway (301). An ascending projection to the habenular nuclei through the fasciculus retroflexus, as well as to the other thalamic nuclei (mediodorsal, parafasciculus, reuniens), has been described (297). Descending projections have been also noted to the dorsal tegmental nucleus and locus coeruleus and diffusely to the pontine reticular formation and caudal central grey (297).

The medial ascending pathway, which primarily innervates the hypothalamus and the preoptic area, originates from the mesencephalic raphe (B7, B8) and pontine raphe areas. The lateral ascending sero-

tonergic pathway, which innervates the cortical areas, originates mainly from the mesencephalic raphe cell groups (B7, B8, B9). The minor, far lateral pathway, which primarily innervates the extra-pyramidal motor system originates from groups B7, B8, B9 (301). The cerebellar innervation originates mainly from the mesencephalic raphe (B7, B8) and possibly from the pontine (B5, B6) raphe cell groups (298).

Biochemical and histochemical studies have clearly indicated the existence of serotonergic pathways, innervating the globus pallidus and the striatum (6,292,302,294). Although there are several reports that lesions involving interruption of the fibres at the level of the ventro-medial tegmentum or of the median forebrain bundle result in extensive reduction of striatal 5-HT and tryptophan hydroxylase (296,303), in more recent studies evidence has been obtained indicating that the MFB does not contain the 5-HT afferents to the striatum (304). Instead, the 5-HT fibres reaching the striatum are postulated to course the area of Tsai at the midbrain and caudal hypothalamic level and then project to the striatum through the rostral part of the cerebellar peduncle and the internal capsule (304).

5.1.5 Nigral and striatal serotonergic innervation

With regard to the particular origin of the serotonergic innervation of the substantia nigra, recent evidence obtained from lesion, autoradiographic and histochemical studies indicates that the DR-B7 and not the MR-B8 is the origin of this projection (305). Furthermore, the area pars compacta of the substantia nigra is believed to be primarily innervated, rather than the area pars reticulata (305). Other workers, however, using electrophysiological and lesion experiments have suggested that the substantia nigra receives a direct monosynaptic inhibitory serotonergic pathway from the MR and not from

DR nucleus (87). It has also been suggested that the 5-HT input to the substantia nigra from the raphe is by way of axon collaterals from a main projection to the striatum (306). Further evidence for a raphe-nigra serotonergic projection comes from a recent report which states that large lesions of the midbrain raphe, which destroy both MR and DR nuclei, decrease significantly the 5-HT levels in the substantia nigra and abolish the potassium-evoked release of endogenous 5-HT from rat nigral slices, probably due to the loss of 5-HT terminals and the resulting loss of tryptophan hydroxylase activity (290).

There is also controversy about the distribution of 5-HT within the substantia nigra. Histochemical studies (6,307), supported by electrophysiological studies (87) suggest that nigral 5-HT is localized mainly in the pars reticulata, but autoradiographic studies using transport of ^{3}H-leucine from the raphe nuclei in the rat and cat (308) indicate that the pars compacta is more heavily innervated than the pars reticulata. Evidence that the pars reticulata has a higher content of 5-HT than the pars compacta (290) is also challenged by another report showing that the two regions have approximately equal 5-HT content (282).

The evidence about the origin of the serotonergic innervation of the corpus striatum is similarly controversial. Although most of the reports agree that the MR (B8) and/or the DR (B7) areas constitute the origin of the main projection from the raphe to the striatum, on the particular raphe cell group giving rise to this projection the evidence is divided. It has been suggested that the striatal serotonergic innervation may originate partly from cells of the B7-B8 area and partly from cells of the group B9 (301). Large electrolytic lesions that destroyed both MR and DR resulted in substantial reduction of striatal

5-HT content (296,289) and reduction in 5-HT synaptosomal uptake and in tryptophan hydroxylase activity (296). Other lesion studies, involving extensive damage of the DR area have showed a reduction of 5-HT in the striatum, whereas lesions of the MR had no effect on striatal 5-HT content (302,309). The same conclusion has been reached from the results of experiments based on the transport of labelled aminoacids, such as ^{3}H-proline or ^{3}H-leucine, by the axonal flow (310) or by the use of the horseradish peroxidase retrograde transport method (302,311). Electrical stimulation of the DR cell group has been found to exert a strong inhibitory action on the firing of striatal cells (302,312), whereas stimulation of the MR was found to inhibit a much smaller percentage of these cells (312).

Contrary to these reports, evidence obtained from lesion experiments indicates that the MR is the main origin of the serotonergic innervation of the striatum. Lesions of the MR cause a fall in striatal 5-HT levels (313,296), in tryptophan hydroxylase activity and in the uptake of 5-HT by synaptosomes prepared from the striatum (296). Furthermore, Costallet al. (314) have reported a fall in 5-HT in the ipsilateral striatum of rats with small electrolytic lesions confined to one side only of the MR. These animals turned away from the side of the lesion after parenteral administration of apomorphine or amphetamine.

5.1.6 Nature of striatal and nigral serotonergic afferents

Despite the controversy over the origin of the nigral and striatal serotonergic innervation, several reports agree about the inhibitory nature of these projections. Electrophysiological experiments indicate that a monosynaptic pathway exists from the DR area to the striatum, whose function is to produce long-lasting inhibition of cell firing in the striatum (302). Inhibition of striatal cells, to a

smaller extent than nigral cells, has also been reported following MR stimulation (312).

Stimulation or lesion of the raphe nuclei produce a marked release or depletion, respectively, of 5-HT in the striatum (318,286,283), suggesting that 5-HT may be a neurotransmitter in this pathway.

5-HT applied directly to cells in the substantia nigra by means of microiontophoresis has little effect on pars reticulata neurons but totally blocks glutamate excitation of pars compacta cells (315). Such evidence suggests that the DAergic neurons in the pars compacta may be the ones primarily influenced by the serotonergic input. Other studies have shown, in agreement with this, that in the substantia nigra the predominant effect of 5-HT is depression of pars compacta cells and both depression and excitation of pars reticulata neurons (64).

Behavioural observations, such as a state of general excitement with increased locomotor activity after selective inhibition of 5-HT synthesis by p-chlorophenylalanine (319,320) or destruction of serotonergic neurons by electrolytic lesions placed in the midbrain raphe (321), point to a participation of 5-HT in the motor function. In general, the 5-HT system in the brain is thought to be an inhibitory one for the locomotor stimulation caused by DA receptor stimulating agents, like apomorphine, or DA releasing agents like amphetamine (272,323). This is further supported by the finding that lesions of the raphe nuclei or inhibition of 5-HT synthesis increase the locomotor activity induced by apomorphine or amphetamine (324,323,322).

5.1.7 Afferents to the raphe: their possible importance

By means of the horseradish peroxidase retrograde transport method, afferents to the midbrain DR and MR nuclei of the rat have been determined by Aghajanian and Wang (325). Only two areas were found to send afferents

to both raphe areas and not to the adjacent reticular formation, the nuclei of the diagonal band and the lateral habenula. The latter area also contained the highest density of afferent cells for both DR and MR (325). The solitary tract nucleus (caudal portion) was found by these investigators to project exclusively to the DR and not to the MR or adjacent reticular formation. The B9 area of serotonergic cells has also been reported to project to the MR (325) whereas it seems that more synaptic interconnections may exist between the raphe nuclei, such as an afferent to the DR from the MR (326,325,327). There is no evidence for the existence of cerebellar afferents to the raphe (325,294,295). The striatum is believed to project to the raphe (328), but another report argues against this idea (325). There is no evidence for afferents from any other area of the basal ganglia or the amygdala (325), whereas there are several reports based on results using various techniques such as horseradish peroxidase, degeneration and autoradiography, for afferents from the preoptic nuclei and the medial forebrain bundle (325,329,330).

Of the major areas that receive a serotonergic innervation from the raphe, e.g. amygdala, hippocampus, septum, basal ganglie, substantia nigra, ventral lateral geniculate, optic tectum and suprachiasmatic nucleus (310,297,6,301), none appears to project directly back to the raphe (325). Thus, there is no close correlation between the raphe efferents and afferents. Among the nuclei projecting to the raphe, the lateral habenula is hypothesized to be a relay nucleus, which, according to Nauta (331) may serve a pivotal role within a 'dorsal pathway' to the midbrain, receiving inputs from amygdala and hippocampus and projecting to the 'limbic midbrain area' via a component of the fasciculus retroflexus, thus regulating or modulating the 5-HT cell neuronal

activity. There is very little evidence about the chemical transmitter used by the described above afferents.

An inhibitory system has been postulated to exist in the raphe, which may have a physiological role of maintaining the slow, regular spontaneous firing of 5-HT neurons by means of 5-HT axon collaterals (316). Also, the discovery that electrical stimulation of the habenular nuclei markedly suppressed the firing of serotonergic neurons in the raphe supports the idea that the habenula may have an important role in regulating or modulating the neuronal activity of serotonergic cells in the midbrain raphe nuclei (317). Attempts to biochemically test the suggestion of Wang and Aghajanian (317) that GABA might be the inhibitory neurotransmitter contained within the habenulo-raphe projection, led to the conclusion that neither GABA nor Ach are likely to fulfill that role (362).

The possibility of a 5-HT axon collateral inhibitory system (316) or 5-HT dendro-dendritic junctions (334) mediating directly inhibition of midbrain raphe neurons after stimulation of the ventromedial tegmentum has also been proposed (316,317), with unknown but perhaps important physiological role in regulating the firing of 5-HT neurons.

In addition to the neuronal feedback mechanisms that may exist, a local feedback system appears to function, in which increased 5-HT availability induced by drugs or 5-HT precursors results in a compensatory decrease of the firing of 5-HT neurons (332). This is not surprising, knowing the high sensitivity of the firing rate of raphe neurons to alterations of 5-HT synthesis, storage, catabolism and re-uptake (235,332).

5.1.8 Feedback mechanisms

Feedback mechanisms triggered by specific receptors control, partly at least, the biosynthesis and release of monoamines in the rat brain. In 1963 for the first time Carlsson and Lindquist (44) observed an increase in the turnover of dopamine in the brain following the administration of dopamine antagonists, i.e. neuroleptics. Similar observations were made later for the other monoamines, such as 5-HT. Thus, stimulation of central 5-HT receptors by LSD (335,344), by 5-methoxy-N,N-dimethyltryptamine (336), by ergocornine (337) and by some hallucinogenic phenylethylamines (338) probably initiates a compensatory negative feedback mechanism which decreases the turnover of 5-HT in brain. In contrast, the blockade of central 5-HT receptors by methysergide (339), by methergoline (340) and by methiothepin (341) induces an increase in the turnover of 5-HT, probably due to the activation of a positive feedback mechanism (342). These feedback mechanisms are still operating in brain slices to control the release of (^{3}H) - 5-HT induced by potassium or electrical field stimulation (343,344).

5.1.9 Catecholamine - serotonin interactions

The presence of nerve terminals and relatively high amounts of 5-HT and tryptophan hydroxylase in areas of the rat brain rich in DA or NA (6,73,282,8) and the existence of catecholamine-containing cells, as well as DA, NA and their synthesizing enzymes in the raphe region in close contact with the 5-HT cells (5,6,282) offer grounds for the postulation of the existence of a physiological interaction between the catecholamines and 5-HT in specific brain structures. Also, fibre bundles belonging to both catecholamines and 5-HT neurons have intimate contacts in the medial

forebrain bundle (8,345). NAergic terminals originating in the locus coeruleus have been observed in the raphe region (346,347), supporting the suggestion that NA may serve as neurotransmitter in the raphe nuclei, with a probable inhibitory function on anterior raphe units, as revealed after electrophysiological experiments (348).

On the basis of biochemical or lesion studies many workers have postulated that for normal motor activity a critical balance should exist between serotonergic and catecholaminergic neurons (350,349). In addition, the cataleptogenic action of various neuroleptics, known to interfere with DAergic transmission, is suppressed by lesions of the raphe which markedly affect striatal 5-HT or by inhibition of 5-HT synthesis with p-chlorophenylalanine (351,352). Increased locomotor response to amphetamine, a catecholamine releasing agent (43,353) is obtained following lesions specific to the MR (354,322) or to the medial forebrain bundle (355) or following the administration of the inhibitor of 5-HT synthesis p-chlorophenylalanine (321). Also, electrical stimulation of the raphe nuclei in rats leads to a decrease in motor activity (321), whereas lesions of these nuclei cause a temporary increase in spontaneous activity (321,356). Apomorphine, a direct DA receptor stimulant (109), increases the brain 5-HT and 5-HIAA, and this effect is antagonised by neuroleptics (357,358), suggesting that this effect is a secondary one, resulting from DA receptor stimulation. The increase in locomotor activity induced by apomorphine is bigger in rats after lesions of the raphe nuclei and after inhibition of 5-HT synthesis (324). The 5-HT system is also thought to be an inhibitory one for the locomotor stimulation induced by amphetamine (272,322,323).

Data suggestive of an interaction between serotonergic neurons of the raphe system and noradrenergic neurons originating in the locus coeruleus are the changes in 5-HT synthesis and/or metabolism following primary catecholamine changes (359,360) or lesions of the locus coeruleus or of the ascending NAergic pathways (361). Electrolytic lesions of the locus coeruleus destroying the NA-containing cell bodies result in a significant increase of 5-HT metabolism in the forebrain of rats (361). The serotonergic innervation of the locus coeruleus has been postulated to have a modulatory influence on the NAergic cell bodies (362).

It is thought that some effects of amphetamine, especially those related to motor behaviour, are strictly related to dopaminergic mechanisms in the striatum (363,353); therefore, the possibility of an interaction between DA and 5-HT should be considered when the effect of this drug is examined in relation to the serotonergic system in the brain. Several other findings point to a participation of 5-HT in the function of the DAergic system. The amphetamine-induced stereotyped behaviour, which is believed to result from an increased availability of DA at striatal receptor sites (363,353) is inhibited by low doses of tryptophan, the 5-HT precursor, and enhanced by methysergide, a 5-HT antagonist (364), whereas neither of these agents appears to affect the intensity or duration of apomorphine-induced stereotypy (365). Moreover, the apomorphine or amphetamine-induced stereotypy is not, probably, significantly affected by mid-brain raphe lesions (366), contrary to the potentiation of the locomotor behaviour described by several investigators (322,354, 324,321).

Large doses of L-dopa produce a marked decrease of brain

5-HT (243,248) and large doses of 5-hydroxytryptophan produce a reduction of DA in the brain (248,269). These actions may be due to displacement of DA by excess 5-HT and vice-versa, or to competition between the exogenous aminoacid and the endogenous counterpart for the synthesising enzymes or for entry into the brain and the neurons (243,248,244). Some behavioural aspects observed after the administration of L-dopa are specifically antagonised by drugs that block serotonergic transmission in the brain (256). Also, agents which are known to block dopaminergic transmission in the brain have been shown to antagonise some behavioural effects of tryptophan (250).

A functional balance between DA and 5-HT in the striatum has been proposed to explain some neurological and pharmacological aspects of human parkinsonism (177). In parallel to DA, 5-HT is reduced in the striatum and substantia nigra of brains from parkinsonian patients, but, unlike DA,levels of 5-HT are above normal after treatment of these patients with MAO inhibitors (117,177).

The available data from animal studies offer satisfactory evidence for a large number of serotonergic fibres reaching the corpus striatum and the substantia nigra. They also point to the idea that these inputs may play an important role in modulating the nigral and striatal function, and consequently may be involved in the regulation of the nigrostriatal dopaminergic pathway. It is possible that 5-HT neurons may impinge on nigrostriatal dopaminergic fibres and nerve terminals or affect the activity of nigral cells and dendrites or influence striatal cells controlled by the dopaminergic input. This would have important physiological and pharmacological significance, in view of the fact that the nigrostriatal pathway has been implicated in very important brain functions.

5.1.10 Statement of the problem,purpose of the present study

The present study was undertaken in order to:

1. Clarify the anatomical origin of the serotonergic projections to the substantia nigra and the corpus striatum of the rat brain. Asymmetric electrolytic lesions were made in specific raphe nuclei and the concentrations of 5-HT and 5-HIAA were measured and compared in the two sides of the substantia nigra and the striatum.

2. Investigate the physiological importance of these projections and, in particular, their influence on the nigrostriatal DAergic pathway. The levels of DA and its main metabolites HVA and DOPAC were determined on each side of the substantia nigra and the striatum following the asymmetric lesions of the raphe nuclei.

3. Investigate possible turning behaviour following asymmetric lesions of the dorsal or the median raphe nuclei. Turning consistent with DA receptor stimulation would indicate that the lesions produced striatal DAergic asymmetry. Various pharmacological treatments were used in order to find the aminergic system or systems responsible for the turning.

4. Examine the possibility of postsynaptic 5-HT receptor 'supersensitivity' induced by the lesion, following the analogy of a similar phenomenon induced by lesions of the nigrostriatal DAergic pathway. The extent of the involvement of the 5-HT and the DA system in the turning behaviour was assessed by measuring the intensity of turning following the treatment of rats with a combination of drugs affecting specifically the individual systems.

5. Consider the hypothesis that the serotonergic projection to the substantia nigra from the raphe nuclei may be part of an alternative mechanism regulating the activity of the nigrostriatal DAergic pathway; to postulate a model for the 5-HT influence on this pathway.

5.2. MATERIALS AND METHODS

5.2.1 Animals

Male albino Wistar rats, weighing 190-210 g at the time of the operation were used throughout the studies. They were housed 3 or 4 to a cage, under automatically controlled temperature and lighting conditions and had free access to food and water. Rats with lesions in the DR were always housed in separate cages from those with MR lesions. The weights of all the animals were regularly measured.

5.2.2 Stereotaxic techniques

Lesions of the median raphe (MR) and the dorsal raphe (DR) areas

Small asymmetric electrolytic lesions of the median or the dorsal raphe area were made. For lesioning, bipolar electrodes, made of two twisted wires 0.15mm in diameter and insulated except for the tip, were used. Lesions were made by passing a DC current for the time necessary for 3 m Coulomb electric charge to cause electrolytic coagulation (about 20-30 sec). The animal was kept anaesthetised with fluothane, circulating through a vinyl mantle over the nose and mouth throughout the operation. After positioning the rat in a David Kopf No. 1530 stereotaxic frame, the head position of the animal was fixed with blunt ear bars, and the electrode was lowered at an angle of 46° (laterally), either into the MR nucleus to co-ordinates A-P 7.8mm, L 4.4mm and V 6.9mm, or into the DR nucleus to co-ordinates A-P 7.8mm, L 5.9mm and V 6.9mm, according to the atlas of Konig and Klippel (14) (A-P: anterior-posterior; L: lateral; V: vertical distance). The lesions were made

routinely in the left side of the desired nucleus.

The bregma suture was used as the stereotaxic reference point for the anterior-posterior and lateral co-ordinates, and the vertical readings were taken from the cortical surface overlying the electrode insertion site. A hole in the skull was opened in the appropriate position, using an electrically driven dental drill. After the completion of the operation, the hole in the skull was sealed with bone wax following the withdrawal of the electrode and an antibiotic powder (puromycin and neomycin mixture) applied locally before suturing the scalp wound with thread.

After operation, the animals were kept warm under an infra-red lamp until they recovered from anaesthesia. They were tested for turning behaviour at least one week and up to four months after the operation. After completion of the behavioural experiments, randomly selected animals were killed and the brains quickly removed. Sections of each brain at the level of the brainstem were cut for histological examination. The remainder of the brain was dissected into areas required for the biochemical assays.

'Sham'-operated control animals (either in DR or in MR) were treated in the same way except that no current was passed after insertion of the electrode to the appropriate co-ordinates.

Positioning the electrode at an angle was found to be essential in order to cause a selective lesion in one raphe cell group without causing damage with the electrode to another group. For example, vertical positioning of the electrode for lesioning the MR cell group would also cause damage to the DR region. Also, when the electrode was positioned vertically at the level of the midbrain area, the sagittal sinus on the cerebral cortex was damaged and the bleeding that

followed made the operation very difficult.

Mortalities among lesioned animals after the operation were very low (less than 5 per cent), but they increased to about 10 per cent after the first postoperative week. Amphetamine-treatment (5 $mg/_{kg}$ i.p.) after the first week increased the death rate. A higher frequency of deaths occurred in rats with the lesion in the MR than in the DR, as follows: MR,9; DR,4.

5.2.3 Behavioural observations

Circling behaviour of lesioned and 'sham'-control animals was measured as the number of complete turns per 30 min to the same direction, performed by each animal after the injection of the appropriate drug (unless otherwise stated). Experiments were always carried out between 11a.m. and 4p.m. For the observation of circling movements the rats were placed in individual plastic circular baths of 50 cm diameter. About 30 min was allowed for each rat to adapt to the new environment and for the diminution of the 'handling effect' (short-lasting, intense circling, which appeared when the rats were handled for the first time) before the test substance was injected. Lesioned animals that did not exhibit at least 30 turns/30 min after a dose of 2 $mg/_{kg}$ i.p. apomorphine were considered as not turning and excluded from further experiments. Only rats turning consistently in one direction were taken into account.

The onset and the duration of turning were always recorded and the intensity of circling behaviour was measured over consecutive 5 min periods. Complete turns to one side of the whole body of the animal were counted. Rats were tested, when necessary, with different drugs, but care was always taken to leave at least a week between injections. Other behavioural observations were noted at the same

time as the measurement of the circling behaviour.

5.2.4 Drugs

DL-Amphetamine sulphate (Sigma Chem. Co.), apomorphine hydrochloride (Mcfarlane and Smith), propranolol hydrochloride ('Inderal', ICI), methysergide bimaleate (Sandoz), phenelzine sulphate (William R. Warner), 5-methoxy-N,N-dimethyl tryptamine (Sigma Chem. Co.) and fusaric acid (Sigma Chem Co.) were dissolved in sterile saline solution (0.9% NaCl). 5-Methoxy-N,N-dimethyl tryptamine was mixed with saline, 2-3 drops HCl 1M were added and heated to 100°C to dissolve completely. Haloperidol ('Serenace', Searle) was commercially provided as a solution in ampoules of 5 mg/ml and the content was diluted with saline to the appropriate concentration. L-Tryptophan was injected as a suspension, prepared as described in Section 4.

All doses were calculated as the free base (or free acid, in the case of acids) and were administered peripherally by the intraperitoneal route (i.p.). All drug solutions were prepared immediately before use. Control animals received the appropriate diluent of the corresponding drug. When two injections of different drugs were given to the same animal, they were administered into opposite sides of the peritoneum.

Details of reagents for the biochemical determinations are given in the description of the particular assay.

5.2.5 Histological assessment of lesion site

Histological analysis of specified sections of brain was made by using a modification of the Klüver and Barrera technique (367). Serial frozen sections of the brain were made on a freezing microtome as follows: After careful removal from the skull, the brain area around

the expected position of the electrode tract was cut (a slice about 4 mm thick) and fixed onto the microtome chuck in a cryostat. The chuck was kept in the cryostat at -40°C until the tissue was frozen and then it was fixed onto the microtome. Coronal sections of 20 µm thick were taken, approximately every fifth one being collected on a glass cover-slip.

Staining was done with luxol fast blue and cresyl violet as follows: Sections were partially dehydrated using 70% ethanol for a few hours and 95% ethanol for 4min. The sections were then stained with luxol fast blue for 30 min (0.1% luxol fast blue in absolute ethanol containing 5 ml of 10% acetic acid per litre). The stained sections were further washed by immersing in distilled water and in 70% ethanol for 1 min. If, after this stage, not only the fibres but the whole section was stained blue, the sections were alternately immersed in 0.05% lithium carbonate solution (1 min) and in 70% ethanol (1 min) until satisfactory differentiation could be observed. Counter-staining was then carried out with 0.1% cresyl violet in 70% ethanol for about 5 min, followed by washing with 95% ethanol for 5-10 min, until satisfactory staining was observed. The doubly stained sections were cleared in xylene for at least 10 min and mounted on slides with canada balsam in xylene.

The fixed sections were observed under the microscope and were compared with illustrations in the stereotaxic atlas of the rat brain of König and Klippel (140). Photographs of sections were made by conventional techniques. Localisation of the lesion site in the appropriate region was a necessary criterion for inclusion of data in this study.

5.2.6 Biochemical determinations

a. Dissection of brain regions

Animals, 2-3 months post operation, were killed by decapitation after a sharp blow on the head. The excised brain was rapidly dissected on an ice-chilled glass plate into four brain regions, as shown in Fig. 1.1.

In this study only the striatum, the substantia nigra and the midbrain area were dissected out (as described in 1.2.1), a procedure that lasted about 2-3 min. The tissue samples of corpus striatum and substantia nigra were frozen immediately in liquid nitrogen, with the left and right side of these tissues immersed simultaneously. Part 4 of the brain was used for the histological examination of the lesioned midbrain area; it included the midbrain area, the pons and the cerebellum. The dissected substantia nigra tissue was about 8 mg from each side and the corpus striatum 40-50 mg from each side. Dissected tissues from turning animals were kept frozen (in liquid nitrogen) and were analyzed within 14 days of dissection, for 5-HT, 5HIAA, DA and NA, HVA and DOPAC.

b. Determination of 5-HT and 5-HIAA

5-HT and 5-HIAA in corpus striatum and substantia nigra were measured by the rapid and sensitive method of Curzon and Green (288). Slight changes in the dilutions of some reagents were made in the present study.

The weighed tissue samples were each homogenized in 3 ml of cold acidified butanol, and, after centrifugation for 10 min at 3,000 revs/min., 2.5ml of the supernatant was pipetted into a glass test tube and shaken mechanically (horizontally, with a Luckham shaker) for 10 min

with 5 ml n-heptane and 0.4 ml 0.1 M HCl containing 0.1% L-cysteine. The two phases were separated by centrifugation for 10 min and 5 ml of the organic phase were transferred into another test tube for the 5-HIAA estimation, and the aqueous phase was used for the 5-HT assay.

0.1 ml of the aqueous phase was mixed in a test tube with 0.6 ml 0.04% o-phthalaldehyde (OPT) in 10 M HCl, vortex mixed and heated in a boiling water bath for 15 min, after which the tubes were cooled in running water. The fluorescence of the solution was measured in microcuvettes using a Perkin-Elmer MPF3 fluorescence spectrophotometer. Activation and fluorescence wavelengths were respectively 360 mμ and 470 mμ (both uncorrected). Standards were prepared as 60 μg/ml solutions in distilled water, diluted 1:100 with 0.1 M HCl (containing 0.1% L-cysteine), and 0.1 ml of this solution was reacted with 0.6 ml 0.04% OPT in 10 M HCl in parallel with the tissue samples. Blanks were prepared by reacting 0.6 ml 0.04% OPT in 10 M HCl with 0.1 ml 0.1 M HCl (containing 0.1% L-cysteine). The peak height was taken as the fluorescence intensity (in arbitrary units).

To measure 5-HIAA, the 5 ml of organic phase, which had been stored in a test tube in ice, was mixed with 0.6 ml 0.5 M phosphate buffer (pH 7.0) and shaken mechanically for 15 min. After centrifugation for 10 min at 3,000 revs/min the organic layer was discarded by aspiration and two 0.2 ml portions of the aqueous phase were pipetted into two 1.5 ml plastic polypropylene (Eppendorf) tubes A and B. To tube A 20 μl L-cysteine 1% was added and to B 20 μl of 0.02% $W/_V$ sodium periodate solution. Then 0.4 ml 10 M HCl was added to both A and B, after which 20 μl 0.1% $W/_V$ OPT in methanol were added to tube B. The tubes were then mixed and placed in a boiling water-bath for 15 min, cooled in water and the fluorescence was read at activation 360 mμ and

fluorescence wavelength 470 mμ.

The peak height of the blank (tube B) was subtracted from that of the test sample (tube A) to give the net 5-HIAA fluorescence. Standards were prepared as 15 μg/ml solutions in distilled water and diluted 1:100 in phosphate buffer pH 7.0, 0.2 ml being added to tubes A and B and processed in parallel with the tubes from tissue samples.

The 5-HT and 5-HIAA content of samples was calculated from curves of fluorescence intensity (in arbitrary units, e.g. mm of peak height) versus the amount of standard 5-HT and 5-HIAA added to tubes and processed in parallel with the samples. The relation between fluorescence intensity and the amount of 5-HT and 5-HIAA present was linear over the range 10-300 ng tested. About 10-15 ng of both 5-HT and 5-HIAA could be detected by this method. Recoveries of both 5-HT and 5-HIAA added to butanol and carried through the procedure were 90-100%. The method was sensitive enough for measuring 5-HT and 5-HIAA in one side of the striatum from one rat brain, but, in order to measure sufficiently both substances in the substantia nigra, left or right sides of this tissue from 2 or 3 brains had to be pooled.

All chemicals were of analytical grade, N-Butanol was acidified by the addition of 0.85 ml concentrated HCl to 1 litre of n-butanol. Solid OPT and L-cysteine (from BDH and Sigma, respectively) were stored at -25°C until use. 0.04% $W/_V$ OPT in 10 M HCl, 0.1% $W/_V$ L-cysteine in 0.1 M HCl, 1% $W/_V$ L-cysteine in distilled water, 0.1% $W/_V$ OPT in methanol and 0.02% $W/_V$ sodium periodate (BDH) solution in distilled water were all prepared immediately before use.

c. Other biochemical determinations

The concentration of dopamine and noradrenaline in each side of the striatum, and the concentration of homovanillic acid (HVA) and

3,4-dihydroxyphenyl acetic acid (DOPAC) in each side of the striatum and of the substantia nigra were measured as described in other Sections of this Thesis.

5.2.7 Statistical analysis

As described in Section 2 (2.2.2. f), for the comparison of turning rates of lesioned rats, paired t test was used, with the rats serving as their own controls against different drug treatments. The turning rate in response to drugs was found to be the same up to 4 months after the operation, supporting the validity of this procedure.

5.3 RESULTS

5.3.1 Animal weights

The weights of the experimental animals, both lesioned and sham-operated, were regularly recorded. As Fig 5.2 indicates, there was no statistically significant difference between the weights of MR- and DR-lesioned animals, or the DR-sham- and the MR-sham-operated animals, or between the DR-lesioned and the DRsham-lesioned animals, up to 80 days after the operation. Paradoxically, there was a significant fall in the mean weight of MR-lesioned animals compared to the MR-sham-lesioned animals during the period of 80 and 100 postoperative days ($p < 0.01$).

5.3.2 Histological assessment of lesion location

The location and the extent of damage in animals with asymmetric electrolytic lesions in the MR and the DR cell body areas are shown in Fig. 5.3. Histological examination of the lesions proved essential to the success of the project. On several occasions the 46° approach had led to the lesion being contralateral to the side of entry of the electrode and a description of the lesion extent was vital in the interpretation of behavioural and biochemical results. Most of the lesions were smaller than the area represented and occasional extension of the lesion along the needle track led to a minimal amount of damage outside the area in some animals.

The extent of the lesioned area at the various planes of the Atlas in about 20 rats with MR and 20 rats with DR lesion was superimposed and

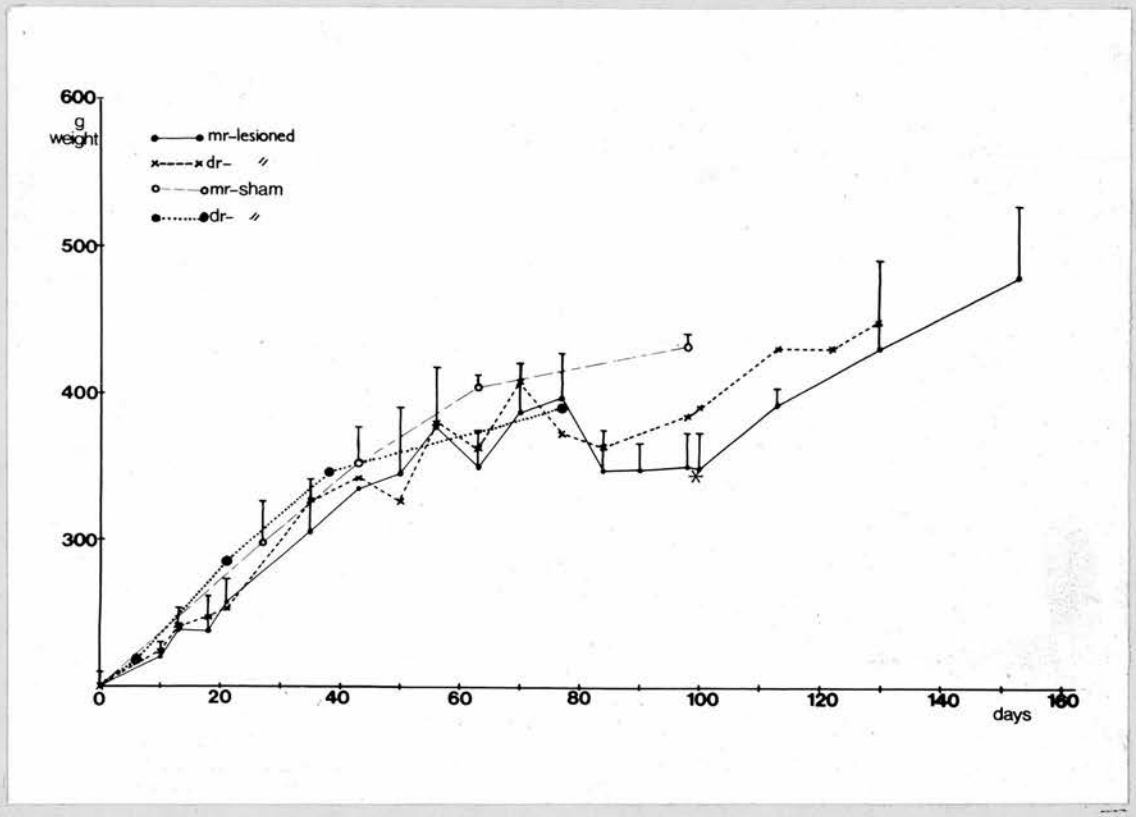


Fig. 5.2

Postoperative development of animal body weight in rats with a lesion in the median raphe (mr) or the dorsal raphe (dr) or sham-operated in either of these nuclei.

Vertical bars represent s.d. of the mean weight obtained from 12-15 rats.

* $p < 0.01$, compared to mr-sham lesioned (control) animals (Student's t test, two-tailed)

the schematic diagram of Fig 5.3 was constructed. Black shading in the drawings represents an area part of which was damaged in all the animals showing turning behaviour.

The extent of the lesions was found to vary slightly between animals, but it was confined primarily between the levels A350 - A160 of the Atlas of König and Klippel (140). However, in most of the brains the damage extended to posterior plane P290 and to anterior plane A620 of the Atlas. The electrode, angled laterally at 46°, caused damage of approximately one-third of the left side of either DR or MR area in each animal. In the group of sham-lesioned animals only the electrode tract could occasionally be observed without any observable damage to the MR or DR nuclei.

Lesions of the dorsal raphe nucleus caused distortion of the aqueductus cerebri (AC) and frequently damaged the fasciculus longitudinalis medialis (FLM) and fasciculus longitudinalis dorsalis pars tegmentalis, the substantia grisea pars ventralis, and very occasionally the pedunculus cerebellaris superior (PCS), but the damage never extended to the areas beneath or to the MR. Lesions of the median raphe nucleus also caused damage to the pedunculus cerebellaris superior (PCS) and tractus tectospinalis and occasionally to the mesencephalic formatio reticularis (FOR) and the nuclei ventralis tegmenti. On rare occasions the lemniscus medialis (LM) was also damaged. The area formatio reticularis (reticular formation FOR) which contains the ascending from the locus coeruleus, dorsal noradrenergic fibres (8) was always carefully examined for any damage. As mentioned above, occasionally lesions of the MR nucleus damaged this area, but there was no correlation between circling behaviour or biochemical changes and the damage to the reticular formation.

Fig. 5.4 represents a diagrammatic representation of the extent

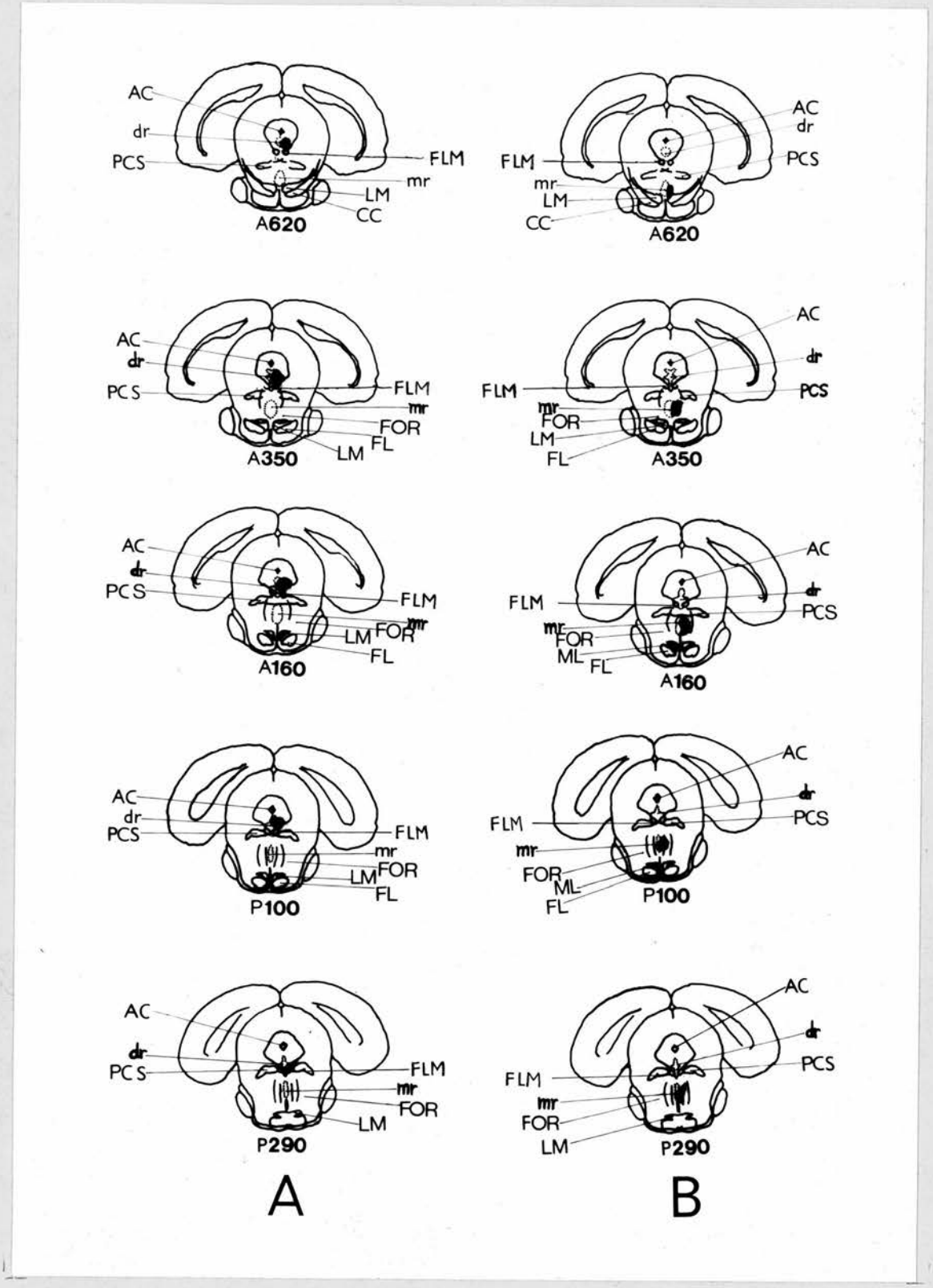


Fig. 5.3

Diagrammatic representation of the extent and localisation of damage caused by lesions placed asymmetrically in A, the dorsal raphe, or B, the median raphe nucleus. The diagrams and coordinates are based upon those of König and Klippel (140). Black shading represents total tissue destruction caused in consistently turning rats, compiled from about 20 rats for each type of lesion.

Abbreviations: AC: aqueductus cerebri; dr: dorsal raphe; PCS: pedunculus cerebellaris superior; FLM: fasciculus longitudinalis medialis; mr: median raphe; LM: lemniscus medialis; FOR: formatio reticularis; CC: crus cerebri; FL: fasciculus longitudinalis

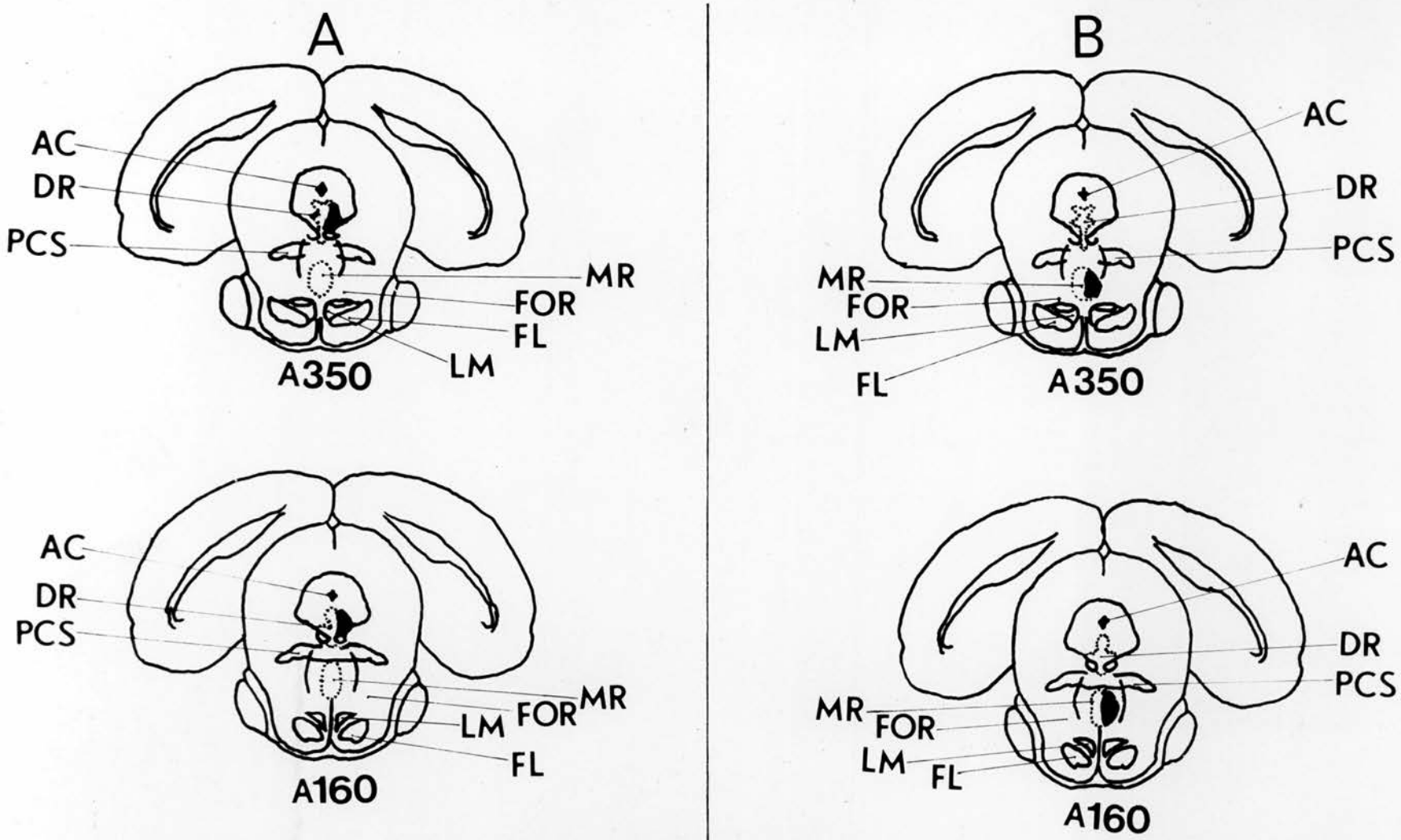


Fig. 5.4

A diagrammatic representation of the localisation and the extent of the damage caused by lesions placed asymmetrically in A, the dorsal raphe nucleus, or B, the median raphe nucleus of the rat

The shaded area represents the total area which was damaged in the 10 animals showing most intense turning behaviour. The most commonly relevant planes are shown.

and the localisation of the damage caused by asymmetric lesions placed in A the dorsal raphe, or B, the median raphe nucleus. The shaded area in the planes A350 - A160 of the König and Klippel Atlas of the Rat Brain (140) represents the area, large part of which was damaged by the lesion in the 10 animals (of each type of lesion) that displayed the most intense turning behaviour.

Fig. 5.5 - 5.11 represent photographs of tranverse histological sections through the midbrain of rats lesioned in DR or MR, with lesions either confined to these areas or damaging other areas adjacent to these two nuclei. The sections were stained with luxol fast blue-cresyl violet, as described in the Methods. Fig. 5.5 shows a photomicrograph of an accurately placed DR lesion. Fig. 5.6 shows a small lesion in the MR nucleus (indicated by the arrow); surrounding cell degeneration and gliosis can be seen. Fig. 5.7 shows a photomicrograph of a DR-lesioned animal the 46° angle approach led to this lesion being contralateral to the side of entry of the electrode and the turning following the administration of apomorphine or amphetamine being in opposite direction compared to typical DR-lesioned animals. The damage due to the passage of the electrode is still visible, at over 80 days after the operation. In Fig. 5.8 a lesion is shown, which damaged the pedunculus cerebellaris superior and part of the reticular formation but not the desired MR nucleus; this atypical lesion did not elicit the behavioural response (circling) that MR-lesioned rats displayed. Fig. 5.9 shows a photomicrograph of another accurately placed DR lesion, and Fig 5.10 and 5.11 represent photographs of sections in posterior planes of the rat brain with small, localised MR lesions (indicated by arrows).

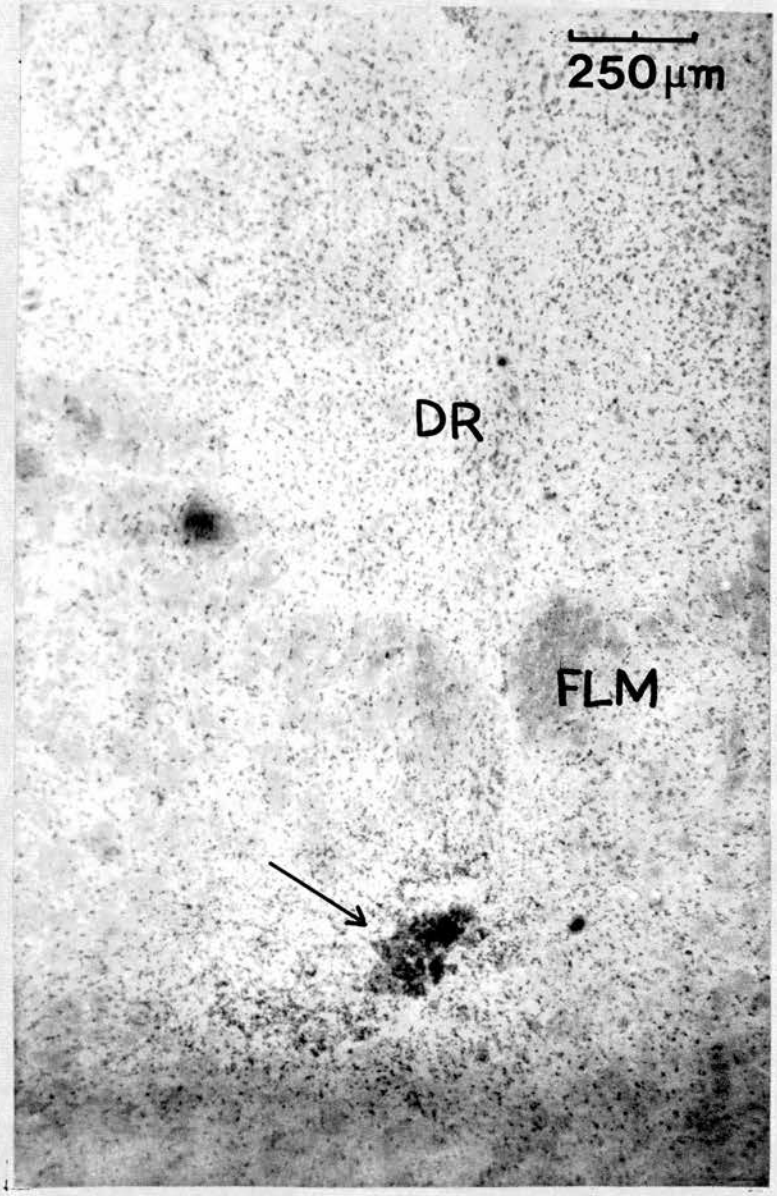


Fig. 5.6

A photomicrograph showing a well-localised asymmetric lesion (arrow) of the median raphe nucleus of the rat; gliosis and cell degeneration in the area surrounding the lesion can be seen. Section stained with cresyl violet-luxol fast blue. (magnification x 80)

Abbreviations: DR - dorsal raphe
FLM - fasciculus longitudinalis medialis

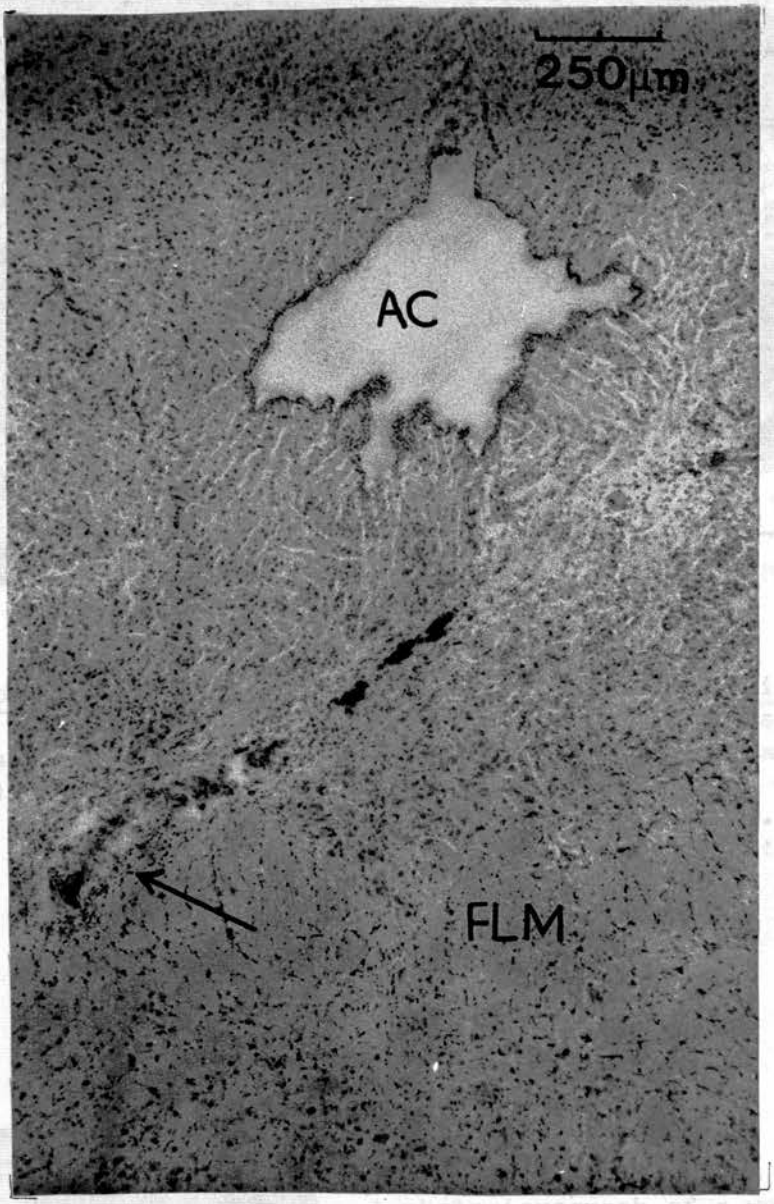


Fig. 5.7

A photomicrograph of a lesion (arrow) in the opposite than the desired side of the dorsal raphe nucleus. The electrode tract can be seen crossing the DR nucleus to the contralateral side. The rat with this lesion responded to apomorphine with turning in opposite direction than typical DR-lesioned rats.

(magnification x 80)

Abbreviations:

AC - aqueductus cerebri
FLM - fasciculus longitudinalis medialis

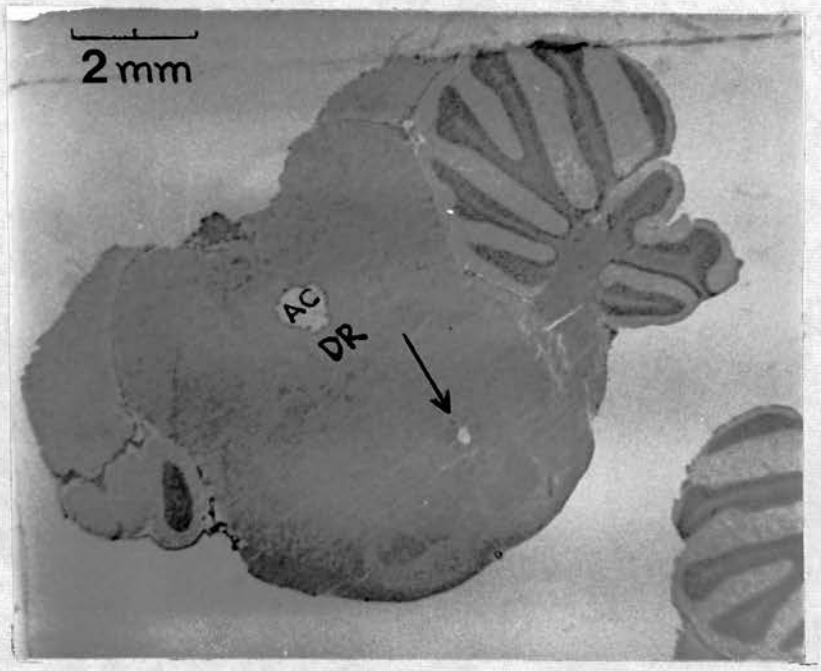


Fig. 5.10

A photograph of a small asymmetric lesion (arrow) of the median raphe nucleus. The section represents a posterior plane of the rat brain. The cresyl violet-luxol fast blue method was used for staining.

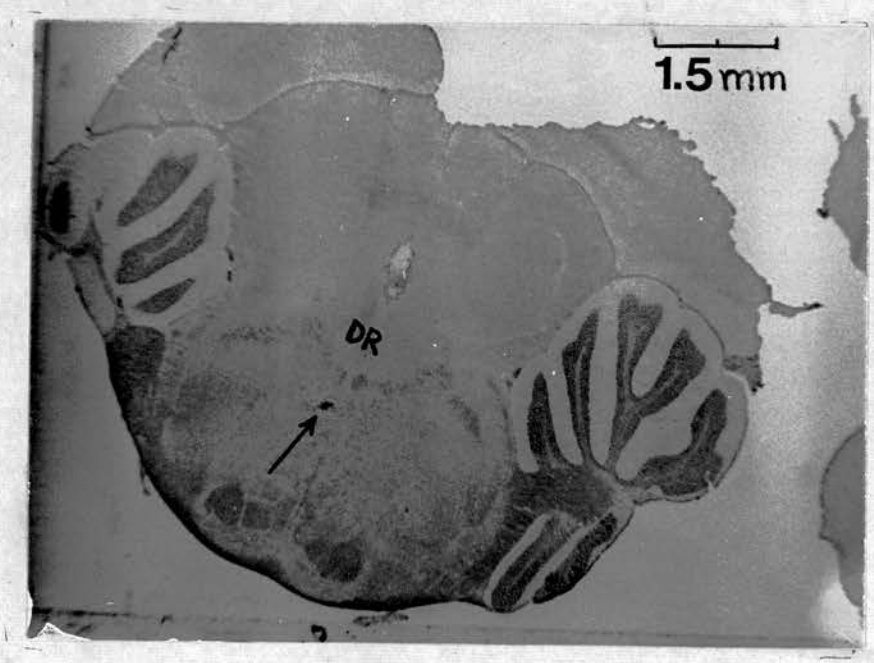


Fig. 5.11

A photograph of a small asymmetric lesion (arrow) in the median raphe nucleus. The section represents a posterior plane of the rat brain. The cresyl violet-luxol fast blue method was used for staining.

5.3.3 Effect of lesions on brain monoamine content

5.3.3a Changes in 5-HT and 5-HIAA after lesions of the raphe nuclei

As shown in the Table 5.1, lesions of left side of MR caused a decrease of 5-HT in the ipsilateral striatum of 34% and a 32% decrease of 5-HIAA levels compared to the contralateral side, both reductions being statistically significant ($p < 0.01$ and $p < 0.025$, respectively, using paired t test). The same lesion resulted in no significant change either in 5-HT or in 5-HIAA in the left substantia nigra when compared to the right substantia nigra (-13% and +10%, respectively).

Contrariwise, lesions of the left side of DR resulted in a 31% reduction of 5-HT and 30% reduction of 5-HIAA in the left substantia nigra when compared to the levels of the right substantia nigra, both changes found with the Student's paired t test significant ($p < 0.01$). In the corpus striatum, the changes in both 5-HT and 5-HIAA in the left side as compared to the right side, were not statistically significant being only -14% and -9% respectively.

The changes in 5-HT and 5-HIAA in the lesioned side of the corpus striatum or the substantia nigra were always of about the same percentage, as the ratios $5\text{-HT}/5\text{-HIAA}$ show (Table 5.2).

Tryptophan loading of rats made the determination of 5-HT and 5-HIAA in small brain areas, like substantia nigra, possible without the need to pool together 2 or 3 tissue pieces from different animals, due to the problem of sensitivity of the assay. Thus an injection of a suspension of L-tryptophan (prepared as described in Section 4) in a dose of 100 $mg/_{kg}$ of animal body weight was given i.p. 1 hour before sacrifice to groups of animals lesioned in the DR or the MR. The animals with asymmetric lesions of the left side of the MR cell group had significantly lower concentrations of 5-HT and 5-HIAA in the left

Table 5.1

Effects of asymmetric electrolytic lesions in the median raphe (MR) or the dorsal raphe (DR) on the concentrations of 5-HT and 5-HIAA in the corpus striatum and the substantia nigra of the rat brain

Lesioned area	Treatment	No. of expts.	Brain region	5-HT Left	5-HT Right	% change	5-HIAA Left	5-HIAA Right	% change
MR	saline	8	CS	0.96 ± 0.22‡	1.45 ± 0.28	-34	0.50 ± 0.11*	0.74 ± 0.14	-32
		5	SN	0.93 ± 0.18	1.07 ± 0.13	-13	1.53 ± 0.32	1.39 ± 0.28	+10
	L-tryptophan	6	CS	1.81 ± 0.28*c	2.37 ± 0.26c	-24	1.60 ± 0.36‡c	2.36 ± 0.45	-32
	100 mg/kg ip	6	SN	1.85 ± 0.48a	2.08 ± 0.46b	-11	3.00 ± 0.54a	2.81 ± 0.32c	+7
DR	saline	5	CS	1.05 ± 0.16‡	1.22 ± 0.22	-14	0.68 ± 0.16‡	0.75 ± 0.08	-9
		4	SN	0.84 ± 0.16‡	1.22 ± 0.14	-31	1.01 ± 0.15‡	1.45 ± 0.22	-30
	L-tryptophan	6	CS	1.99 ± 0.20c	2.30 ± 0.37b	-14	2.14 ± 0.17*c	2.63 ± 0.35c	-19
	100 mg/kg ip	6	SN	1.03 ± 0.19‡‡	2.29 ± 0.35b	-55	2.58 ± 0.45‡c	3.47 ± 0.43c	-26
Sham MR	saline	4	CS	1.34 ± 0.16	1.39 ± 0.11		0.65 ± 0.13	0.72 ± 0.14	
		4	SN	1.20 ± 0.20	1.33 ± 0.34		1.30 ± 0.34	1.20 ± 0.26	
Sham DR	saline	4	CS	1.43 ± 0.23	1.46 ± 0.33		0.76 ± 0.08	0.70 ± 0.12	
		4	SN	1.11 ± 0.19	1.04 ± 0.12		1.30 ± 0.19	1.41 ± 0.24	

5-HT and 5-HIAA are expressed as µg/g tissue ± s.d. The content of the right or contralateral to the lesion and left or ipsilateral to the lesion side of corpus striatum (CS) and substantia nigra (SN) of the rat brain was determined in sham-lesioned, in MR-lesioned and DR-lesioned animals, both after saline and 1 hour after an injection of 100 mg/kg L-tryptophan suspension i.p. Tissue weights were about 8 mg for substantia nigra and 40-50 mg for striatum (each side).

All rats with lesions were tested for turning and sacrificed about 2-3 months after the operation.

Significance (paired t test): *: $p < 0.005$; **: $p < 0.025$; ‡: $p < 0.01$; ‡‡: $p < 0.001$ (compared to the other side)

Student's t test (two-tailed): a: $p < 0.01$; b: $p < 0.005$; c: $p < 0.0025$ (compared to the same side in saline-treated lesioned animals)

Table 5.2

Effect of raphe lesions on the ratio 5-HT/5-HIAA

Lesioned area	Treatment	No. of expts.	Brain region	5-HT/5-HIAA Left	Right
MR	saline	8	CS	2.14 ± 0.49	2.00 ± 0.37
		5	SN	0.72 ± 0.20	0.79 ± 0.10
	L-tryptophan 100 mg/kg ip	6	CS	1.38 ± 0.49	1.04 ± 0.22
		6	SN	0.62 ± 0.19	0.73 ± 0.12
DR	saline	5	CS	1.60 ± 0.30	1.62 ± 0.24
		4	SN	0.85 ± 0.23	0.87 ± 0.18
	L-tryptophan 100 mg/kg ip	6	CS	0.93 ± 0.09	0.88 ± 0.12
		6	SN	0.44 ± 0.18*	0.69 ± 0.28

Asymmetric electrolytic lesions in the median raphe (MR) or the dorsal raphe (DR) were made and the ratio 5-HT/5-HIAA in the corpus striatum (CS) and the substantia nigra (SN) of the rat brain was calculated.

The ratios 5-HT/5-HIAA were calculated from the data of Table 5.1. Results represent means ± s.d.

Significance (Student's t test for paired data): $^{*}p < 0.001$ (compared to the right side)

side of the corpus striatum than in the right side ($p < 0.05$ and $p < 0.01$, respectively, paired t test). Both sides had significantly higher concentrations of 5-HT and 5-HIAA than the corresponding sides of saline-treated animals. The difference between the mean values of the two sides was -24% for 5-HT and -32% for 5-HIAA. There was no statistically significant difference between left and right substantia nigra in either 5-HT or 5-HIAA concentration, the difference between the mean values being -11% and +7%, respectively (Table 5.1).

An injection of L-tryptophan to rats with a lesion in the left DR cell group resulted in a statistically significant rise in 5-HIAA in both sides of the substantia nigra (left +157%. right +139%), whereas only the right (contralateral) side showed a significant rise in 5-HT (+98%), the 23% rise of the left (ipsilateral) subtantia nigra not resulting in a significantly different concentration compared to the corresponding side of saline-treated, lesioned animals. Therefore, the concentration of 5-HT in the left substantia nigra was lower by 55% ($p < 0.001$, paired t test) whereas the concentration of 5-HIAA was only lower by 26% ($p < 0.01$, paired t test) compared to the contralateral side.

In the corpus striatum (where 5-HT and 5-HIAA were reduced after lesions of the MR and not of the DR) L-tryptophan treatment of rats with an asymetric DR lesion caused a smaller increase in 5-HT and 5-HIAA in the side ipsilateral to the lesion than in the contralateral side. Thus, the concentration of 5-HT and 5-HIAA was significantly higher in both sides of the corpus striatum, compared to the corresponding sides in saline-treated animals with a DR lesion (Table 5.1), but the 5-HT and 5-HIAA were lower in the ipsilateral compared to the contralateral side (by 14% and 19%, respectively); the difference was statistically

significant only with respect to 5-HIAA ($p < 0.025$, paired t test).

Sham-lesioned animals, either in the DR or in the MR, showed no statistically significant difference in 5-HT or 5-HIAA between the two sides of the corpus striatum or the substantia nigra. Furthermore, there was no significant difference between the 5-HT or 5-HIAA concentrations in the side of the corpus striatum or the substantia nigra contralateral to the lesion in the DR or MR and the concentrations in either side of rats with sham-lesions (Table 5.1).

A comparison of the ratios $5\text{-HT}/_{5\text{-HIAA}}$ in the left and the right side of corpus striatum and substantia nigra (Table 5.2) shows that the lesions, in general, did not cause any change, except on one occasion: in the substantia nigra of rats with lesions in the DR, after treatment with L-tryptophan. In this case, a significant reduction of the ratio was found ($p < 0.001$, paired t test), due to the smaller increase of 5-HT in the lesioned side after L-tryptophan (+23%) compared to the 98% increase in the right side, whereas 5-HIAA concentrations increased by approximately the same percentage in both sides (left: +157%; right: +139%).

5.3.3b Effects of the lesions on catecholamine metabolism

There was no statistically significant change in the concentration of either NA or DA in the left (ipsilateral) side of the striatum compared to the right (contralateral) side of the same structure in rats with lesions of the left DR or the left MR nucleus (Table 5.3). The sensitivity of the radiometric assay did not allow the measurement of the two amines in the substantia nigra.

The concentrations of the main metabolites of DA in brain, i.e. homovanillic acid (HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC) were determined in the two sides of the corpus striatum and the substantia

Table 5.3

Effects of median raphe (MR) or dorsal raphe (DR) lesions on catecholamine metabolism

Lesioned area	Brain Region	Dopamine		Noradrenaline	
		Left	Right	Left	Right
MR	CS	4.468 ± 0.534(4)	4.421 ± 0.677(4)	0.631 ± 0.063(4)	0.688 ± 0.130(4)
DR	CS	3.760 ± 0.306(4)	3.956 ± 0.451(4)	0.678 ± 0.147(4)	0.558 ± 0.089(4)
		HVA		DOPAC	
MR	CS	1.37 ± 0.14(9)**	0.94 ± 0.14(9)	1.81 ± 0.29(9)**	1.35 ± 0.22(9)
	SN	1.48 ± 0.28(8)	1.61 ± 0.32(8)	1.46 ± 0.27(8)	1.37 ± 0.34(8)
DR	CS	0.96 ± 0.13(6)	0.97 ± 0.12(6)	1.37 ± 0.12(6)	1.44 ± 0.21(6)
	SN	2.93 ± 0.64(5)*	1.61 ± 0.28(5)	2.23 ± 0.39(5)*	1.05 ± 0.10(5)

The effects of asymmetric electrolytic lesions in the MR or the DR on dopamine and noradrenaline concentrations in the corpus striatum (CS) and on homovanillic acid (HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC) levels in the corpus striatum (CS) and the substantia nigra (SN) of the rat brain are shown. The lesion was in the left side of the raphe nuclei.

Results are expressed as μg/g of tissue weight ± s.d; number of animals is in parentheses.

The amine and metabolite levels were determined as described in the Methods. All the animals were sacrificed about 2–3 months after the operation.

Statistical significance (Student's t test for paired samples): * $p < 0.005$; ** $p < 0.001$

The weight of tissue analyzed was about 8mg for substantia nigra and 40 – 50mg for corpus striatum (each side)

nigra by the sensitive gas chromatographic technique described in the Appendix. The results are shown in Table 5.3.

An asymmetric electrolytic lesion of the MR which affected only the left side of this nucleus and resulted in a significant reduction of 5-HT and 5-HIAA in the ipsilateral striatum (Table 5.1), also caused statistically significant rises in HVA and DOPAC in the ipsilateral striatum compared to the contralateral side ($p < 0.001$, paired t test). No statistically significant difference in either HVA or DOPAC was found between the left and the right substantia nigra, in good correlation with the 5-HT and 5-HIAA, which did not change in the left compared to the right side of this structure after asymmetric MR lesions.

The concentrations of HVA and DOPAC were determined in the left and the right substantia nigra and in the left and the right striatum in a group of successfully rotating animals with a lesion in the left side of the DR nucleus. No significant difference was found between the two sides of the corpus striatum in the concentrations of these metabolites, whereas there was a highly significant rise in both HVA and DOPAC in the left compared to the right substantia nigra ($p < 0.005$, paired t test). Thus, an inverse correlation was found in the substantia nigra between the changes induced by DR lesions in 5-HT and 5-HIAA (Table 5.1) and HVA and DOPAC (Table 5.3).

5.3.4 Circling behaviour and other behavioural observations

Both in the acute and the chronic postoperative period, the electrolytically lesioned rats displayed slow spontaneous turning lasting 1-2 min and a tendency to unilateral turning of the head immediately after they were placed into the plastic circular bowl. Rats with asymmetric lesions in the MR usually turned to the right

(contralateral) side, whereas those with asymmetric lesions in the DR turned to the left (ipsilateral) side; however, a number of DR-lesioned animals did not exhibit any spontaneous turning. The spontaneous behaviour (turning and postural asymmetries) was more pronounced during the first postoperative week, becoming less obvious and finally disappearing in the 3-4 months after the operation. Spontaneous circling is common in other types of neurological lesions, such as 6-hydroxy-dopamine - induced lesions of the substantia nigra (8) and has been attributed to handling effect. In general, a gross comparison with sham-operated control rats showed an increased excitability and hyperactivity in both MR- and DR-lesioned animals, in agreement with several published reports (368,321,356).

5.3.4a Apomorphine and amphetamine effects

Apomorphine, a drug thought to directly stimulate DA receptors (109) enhanced the spontaneous turning and induced turning in rats not turning spontaneously. The rate of turning was dose-related (as shown in the Fig. 5.12) between 0.5 - 5 $mg/_{kg}$ body weight. After a dose of 2 $mg/_{kg}$ i.p. the turning started in 2-3 min, continued for about 30 min at a constant rate and lasted usually 45 min (with more than one complete turn per minute). Not infrequently the turning lasted up to 2 hours, especially in MR-lesioned rats. Fig. 5.13 shows a typical raphe (MR or DR)-lesioned rat, turning after apomorphine administration.

In MR-lesioned animals apomorphine in doses 0.5 - 5 $mg/_{kg}$ i.p. caused contralateral turning, as reported by Costall and Naylor (368), which was slower than the turning reported by the above authors. It was however, found consistently in rats which at the histological assessment showed that they had the lesion properlylocated in the left side

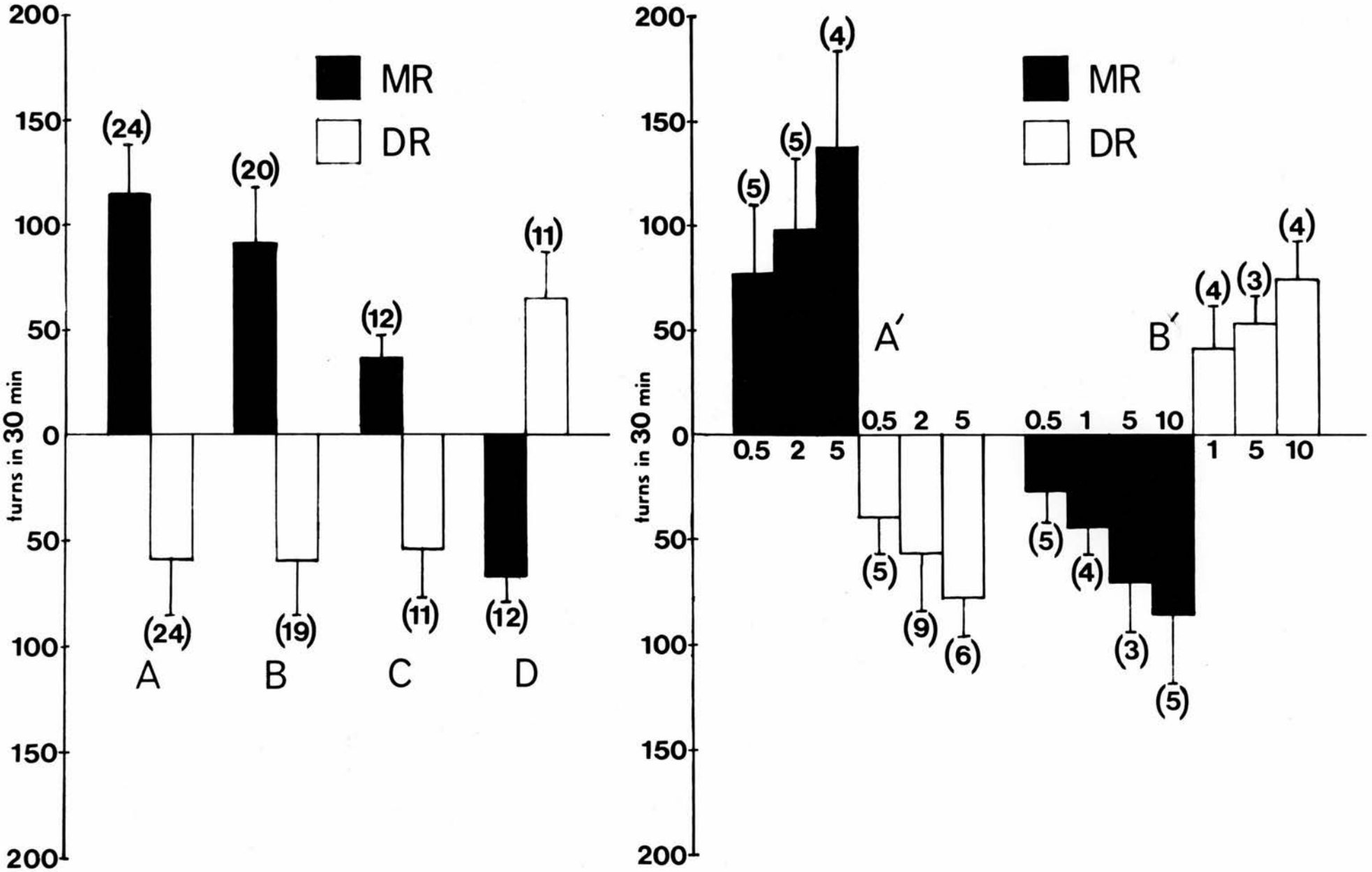

200
150
100
50
0
50
100
150
200
turns in 30 min
MR
DR
(24)
(20)
(12)
(11)
(24)
(19)
(11)
(12)
A
B
C
D
(4)
(5)
(5)
A′
B′
(4)
(3)
(4)
0.5
2
5
0.5
1
5
10
0.5
2
5
1
5
10
(5)
(9)
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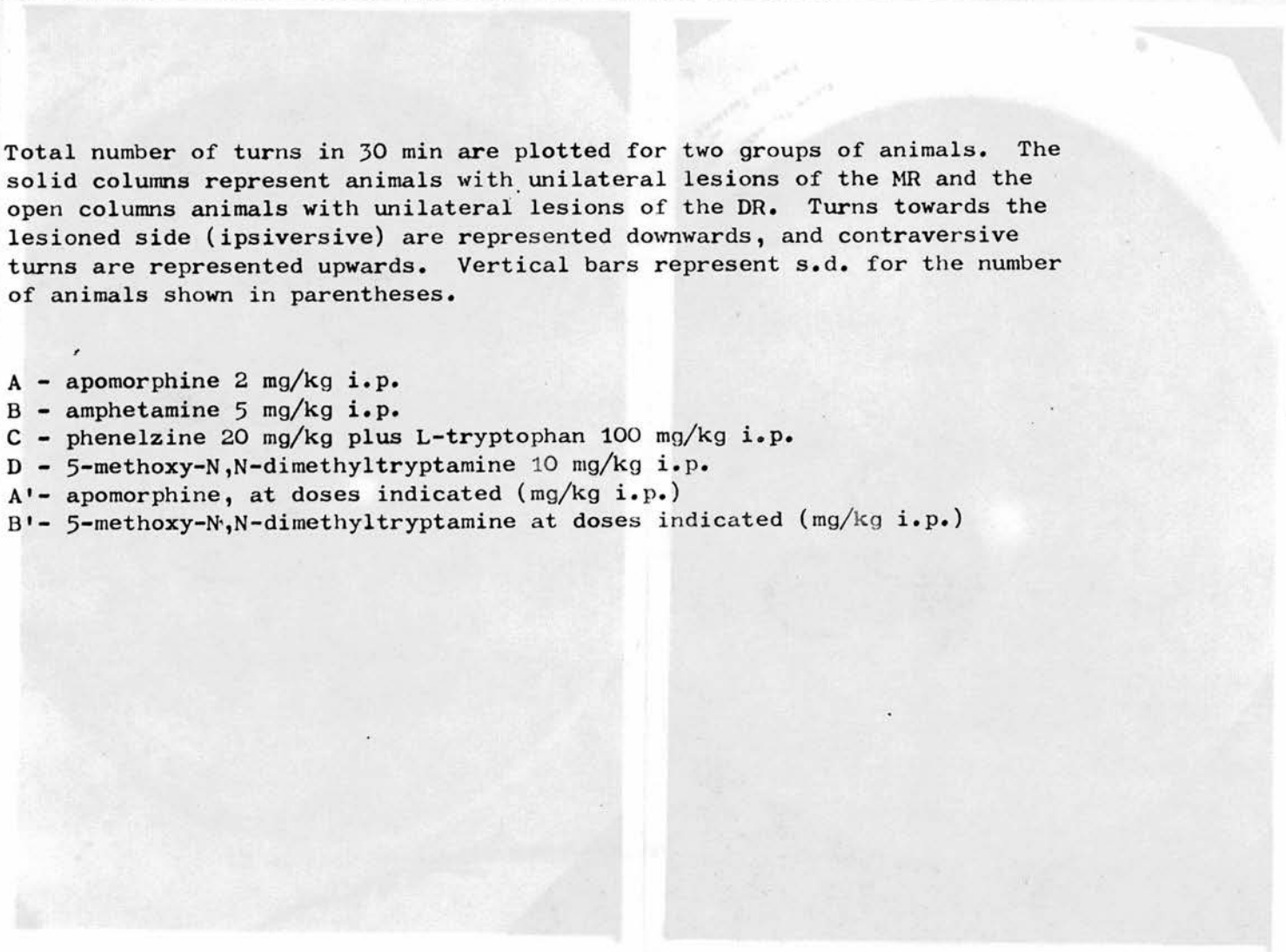
Fig. 5.12

Fig. 5.12

Turning behaviour of rats with a lesion in the median or the dorsal raphe.



Total number of turns in 30 min are plotted for two groups of animals. The solid columns represent animals with unilateral lesions of the MR and the open columns animals with unilateral lesions of the DR. Turns towards the lesioned side (ipsiversive) are represented downwards, and contraversive turns are represented upwards. Vertical bars represent s.d. for the number of animals shown in parentheses.

A - apomorphine 2 mg/kg i.p.
B - amphetamine 5 mg/kg i.p.
C - phenelzine 20 mg/kg plus L-tryptophan 100 mg/kg i.p.
D - 5-methoxy-N,N-dimethyltryptamine 10 mg/kg i.p.
A'- apomorphine, at doses indicated (mg/kg i.p.)
B'- 5-methoxy-N,N-dimethyltryptamine at doses indicated (mg/kg i.p.)

of the MR (as shown in Fig. 5.6, 5.10, 5.11). A relatively slower turning was obtained when apomorphine (0.5 - 5 mg/kg i.p.) was given to rats that had an asymmetric lesion in the DR nucleus, but the animals turned consistently to the ipsilateral side. The rate of turning was also dose-dependent, as Fig. 5.12 illustrates.

In both types of lesion, the rats turned in tight circles, (Fig. 5.13), frequently showing half turns to one side followed by complete turns to the other. No difference could be observed in the way the animals turned in the MR or DR type of lesion, apart from the direction of turning and the difference in the rate of turning. In both lesions, the rats responding to apomorphine were turning in the centre of the circular bath, within a small radius, usually tending to pivot round the hind limbs. About 80% of the lesioning operations were successful in producing animals responding to apomorphine with circling behaviour.

Sham-operated animals showed no consistent postural asymmetrics or spontaneous turning. They did not show any turning response to apomorphine or amphetamine. During the first week after lesioning, mild asymmetric postures of the head or the body, probably of neurological nature, were observed, but subsequently disappeared.

Locomotor stimulation and body asymmetry were more pronounced after apomorphine. Also hyperactivity, stereotyped movements (such as pacing and padding of the forepaws, sniffing, licking, knawing), tail stiffening or tail erection, clinging with the hindpaws on the walls of the bath and even sidewards jumping were common characteristic effects of apomorphine administration (0.5 - 5 mg/kg i.p.), observed in MR- and DR-lesioned rats and in the control sham-lesioned rats. Appearance of these effects, especially the sniffing and the stereotyped

movements were taken as an indication that the animals responded to apomorphine, whereas in the absence of these symptoms the injection was considered unsuccessful and was repeated.

When given in a dose of 5mg/kg i.p., DL-amphetamine, an agent believed to be acting indirectly on DA receptors in the brain by releasing DA from the nerve terminals (43), caused intense turning. The turning started at 3-4 min after the injection and lasted for about 45 min at a constant rate, and continued at a slowering rate for as long as 90 or 120 min. The intensity of turning was in general higher in rats with a lesion in the MR than in rats with a lesion in the DR. Rats with an asymmetric lesion in the MR responded to amphetamine with intense turning contralateral to the side of the lesion. Rats with an asymmetric lesion in the DR responded to the same treatment with turning ipsilateral to the side of the lesion (Fig. 5.12). No turning was apparent after amphetamine administration to sham-lesioned animals.

Both in sham-operated and in MR- or DR-lesioned rats, amphetamine administration was followed by hyperactivity and increased locomotion, as shown by the intense stereotyped and exploratory behaviour. Also, piloerection, clinging on the walls of the circular bath, tail erection, movements of the head, exploration of the air-space with the head, sniffing, and, occasionally, jumping, backwards movements, standing on the hindpaws were the behavioural effects consistently observed after amphetamine.

5.3.4b Effect of haloperidol on apomorphine- or amphetamine-induced turning

The DA receptor blocking agent haloperidol, at doses of 0.5 or 1.0mg/kg i.p. given 1 hour before the injection of apomorphine or amphetamine, inhibited the turning induced by these agents, in both

MR- and DR-lesioned rats. When it was injected after the administration of either of the two drugs, haloperidol inhibited the rotation of the rats within 5 min. No turning was observed when haloperidol alone was administered.

Mild to severe catalepsy with subsequent disappearance of the spontaneous movements, proptosis of the head, sedation and flattening of the body, with extended paws, squeaking (when the animals were touched) were the characteristic behavioural effects of haloperidol. Most of the previously described behavioural effects of apomorphine were eliminated in rats pretreated with haloperidol. No sniffing or stereotyped movements were observed, but the postural asymmetries of the rats persisted even after haloperidol administration. Apomorphine ($2mg/_{kg}$ i.p.) administered after haloperidol did not alter the cataleptic effect of this drug, whereas haloperidol given after apomorphine induced catalepsy within 5 min (in addition to the interruption and inhibition of turning).

Haloperidol ($0.5mg/_{kg}$ or $1mg/_{kg}$ i.p.) also caused catalepsy when it was given to rats either 1 hour before or within the 30 min after the administration of amphetamine ($5mg/_{kg}$ i.p.), in addition to the complete inhibition of turning. The rats were sedated, alert but not moving and squeaking when touched. Complete disappearance of the stereotyped movements were observed, but an interesting exploratory behaviour was also noticed; the animals were only moving their heads, exploring the air-space. The postural asymmetries induced or enhanced by amphetamine also persisted when haloperidol was administered.

5.3.4C. Effects of L-tryptophan-phenelzine drug combination

L-tryptophan, the 5-HT precursor, at a dose of 100mg/kg i.p. and following the injection of the MAO inhibitor phenelzine sulphate given at a dose of 20mg/kg i.p. 1 hour earlier, caused contralateral turning in rats with a MR lesion and ipsilateral turning in rats with a DR lesion, (Fig. 5.12). About 80% of the MR- and DR-lesioned rats that turned with apomorphine also turned in the same direction with this drug combination. An even higher percentage (90%) of the rats that turned after amphetamine responded with circling behaviour in the same direction after the combination phenelzine-tryptophan (Table 5.4). The turning was slow in onset and rate: it was starting between 30-60 min after tryptophan injection and lasting for about 2 hours. No circling was observed with phenelzine or with tryptophan alone.

The typical behavioural symptoms of this drug combination were always observed: Flattening of the body, compulsive sniffing, squeaking and excitability, padding of the forepaws, movements of the head, shivering, pilo- and penile-erection, salivation, proptosis of the head. Postural asymmetries persisted throughout the duration of action of this drug combination. Marked sedation was induced by phenelzine alone, but most of the above effects appeared only after the administration of tryptophan. The rats had periods of deep sedation and interruption of turning, so that the actual intensity of turning after subtraction of these dead-time intervals could be much higher than the intensity shown in the Fig. 5.12.

5.3.4d Effects of 5-methoxy-N,N-dimethyltryptamine

The drug 5-methoxy-N,N-dimethyltryptamine (5-MDT) thought to be a central 5-HT receptor stimulant, was injected at a dose of 10mg/kg i.p. and caused ipsilateral turning in rats with an asymmetric lesion in the MR and contralateral turning in rats with an asymmetric lesion in the DR, i.e. in the opposite direction than apomorphine or amphetamine (Fig. 5.12). The turning rate was dose-dependent in the range 0.5-10mg/kg i.p. tested, as Fig. 5.12 illustrates. All rats that had responded to apomorphine and amphetamine, responded also with circling behaviour after administration of this agent. A peculiar and very characteristic way of turning was observed with this drug (Fig. 5.14). Two to three min. after the injection, the body was flattened completely and turning started, with great difficulty in moving. Instead of turning actively about on one hind leg as they did after amphetamine or apomorphine, the animals were turning with all their legs extended laterally, and their abdomen close to the surface of the bowl. Shivering and convulsions of the body were observed, with padding of the forepaws and thehindpaws extended and also elongation of the body. No exploration, stereotypy or marked hyperactivity was observed.

5.3.4e Correlations between circling responses

As shown in Table 5.4., apomorphine (2mg/kg i.p.) and DL-amphetamine (5mg/kg i.p.) caused contralateral turning (away from the lesioned side) in 22 out of 25 rats with an asymmetric lesion in the MR and in opposite directions in the other 3 rats. Among the DR-lesioned rats, these two drugs induced ipsilateral turning (towards the lesioned side) in 18 out of 19, and in opposite directions in 1 rat.

DL-Amphetamine and the combination phenelzine plus L-tryptophan induced turning in the same direction in 11 and in opposite direction in

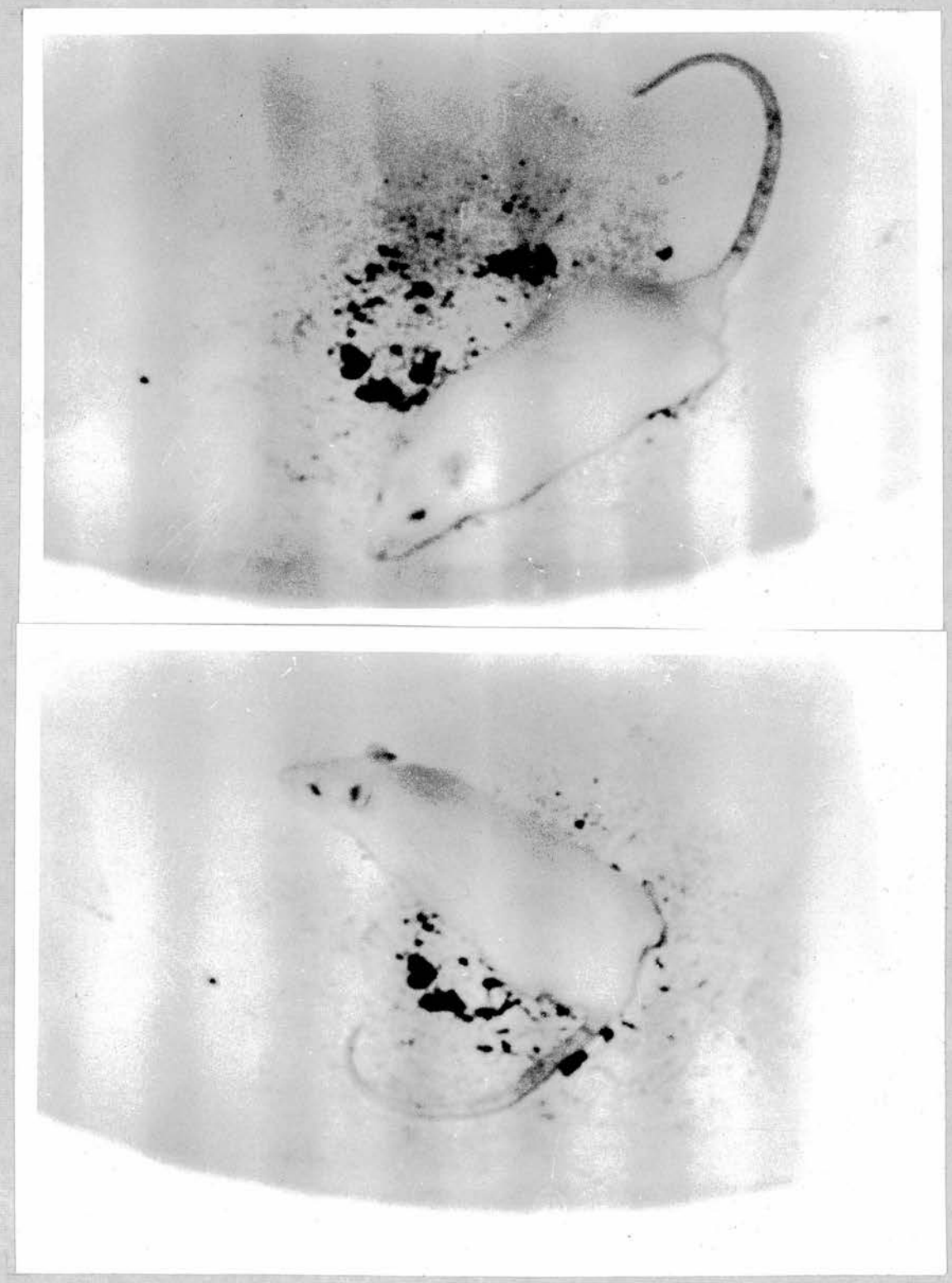


Fig. 5.14

The turning rat with a raphe lesion following the administration of 5-methoxy-N,N-dimethyltryptamine.

Table 5.4

Correlations between directions of circling responses of rats with median raphe (MR) or dorsal raphe (DR) lesions

Drugs \ Direction	same		opposite	
	MR	DR	MR	DR
apomorphine Vs DL-amphetamine	22	18	3	1
apomorphine Vs phenelzine plus L-tryptophan	14	15	2	3
apomorphine Vs 5-MDT	-	1	5	6
DL-amphetamine Vs phenelzine plus L-tryptophan	11	19	2	-
DL-amphetamine Vs 5-MDT	1	1	6	7

Drugs were administered to rats at intervals of about 10 days, at doses described in the text. The numbers of rats tested are indicated.

2 of the MR-lesioned rats; in the DR-lesioned rats, the direction of turning was the same in all 19 rats tested, i.e. ipsilateral to the side of the lesion.

Apomorphine and the combination phenelzine plus L-tryptophan induced contralateral turning, in MR-lesioned rats, in 14 out of 16 animals, with the other 2 turning in opposite directions. In DR-lesioned rats, the turning was ipsilateral in 15 out of 18 rats, with the other 3 turning in opposite directions.

When compared with apomorphine, 5-MDT caused circling in the opposite direction in all 5 rats tested that had an asymmetric lesion in the MR. Of the 7 rats with an asymmetric lesion of the DR that were tested, 1 turned in the same direction and 6 in opposite directions with the two drugs.

All correlations of the directions of turning are shown in Table 5.4.

5.3.4f Acute and chronic effects of haloperidol on circling

As mentioned above, pretreatment of rats with 0.5 or 1mg/kg haloperidol inhibited completely the turning induced by apomorphine (2mg/kg i.p.) or amphetamine (5mg/kg i.p.). However, when haloperidol was administered chronically for 10 or 15 days, at a dose of 1mg/kg i.p. daily, tolerance developed to the blocking effect of this drug on the circling induced by apomorphine or amphetamine. Apomorphine (2mg/kg i.p.) administered 24 hours or 1 hour after the last (15th) injection of haloperidol to rats lesioned in the MR elicited turning that was significantly more intense ($p < 0.05$ and $p < 0.025$, respectively, paired t test) and in the same direction, compared to the turning recorded when the same animals were tested with apomorphine alone, without haloperidol pretreatment (Fig. 5.15). Similarly, amphetamine (5mg/kg i.p.),

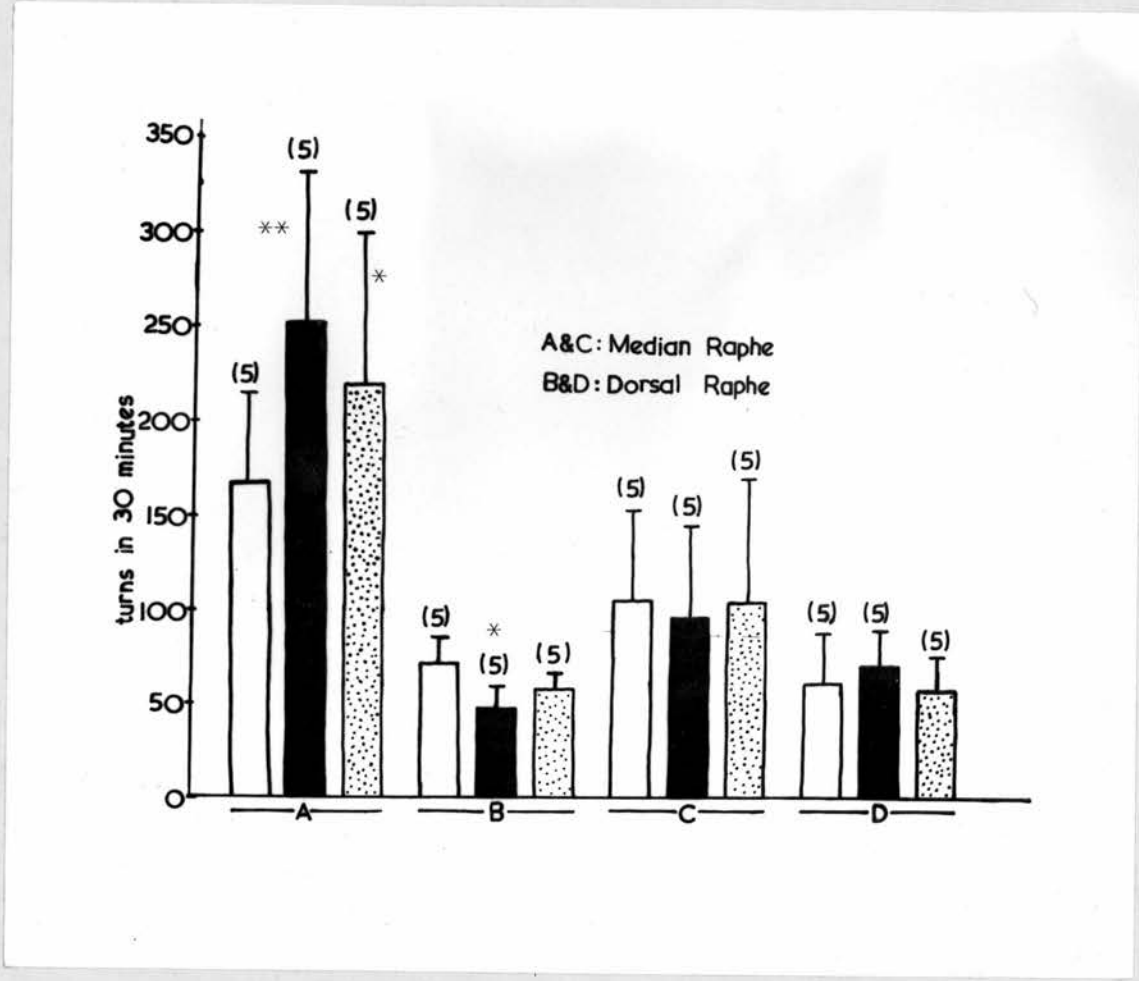


Fig. 5.15

Modification of turning behaviour by chronic haloperidol treatment

Open columns represent turning after 2 mg/kg apomorphine (A and B) or 5 mg/kg amphetamine (C and D). Filled columns represent turning after apomorphine (A and B) or amphetamine (C and D) given 1 hour after the last of 15 daily injections of haloperidol (1 mg/kg daily). Dashed columns represent the responses to the same treatments 24 hours after the last (15th) injection of haloperidol.

* $p < 0.05$
** $p < 0.025$
(paired t test)

when administered to MR-lesioned rats after pretreatment for 15 days with haloperidol (1mg/kg i.p. daily), elicited turning which was of about the same intensity and in the same direction as the turning obtained when the same animals were injected with amphetamine alone (without pretreatment).

In rats with an asymmetric lesion in the DR, apomorphine caused significantly less intense turning ($p < 0.05$, paired t test) at 1 hour but not at 24 hours after the last injections of haloperidol and always in the same direction. Amphetamine caused turning of about the same intensity and in the same direction in rats with a DR lesion, when it was administered 1 hour or 24 hours after the last of 15 daily injections of haloperidol, compared to the response obtained when it was administered alone (without pretreatment). All the above results are illustrated in Fig. 5.15.

With regard to the other behavioural effects of apomorphine (2mg/kg i.p.), both 1 hour and 24 hours after the last of a series of 10 or 15 daily injections of haloperidol (1mg/kg i.p.) to rats lesioned either in the MR or the DR, these were more intense than the effects following a single apomorphine dose without the chronic pretreatment with the DA receptor blocker. Stereotyped repetitive, apparently purposeless movements (sniffing, gnawing, licking, padding of the forepaws, clinging on the walls of the bath with the forelimbs), postural asymmetries, tail erection, occasionally backwards movements and general excitability were the behavioural symptoms common to all rats that had undergone this treatment. The circling continued for about 45 min and was followed by sedation and mild catalepsy, with interruption of turning and disappearance of the apomorphine effects (sniffing, stereotyped movements, tail erection, etc). Catalepsy also

developed if haloperidol (1mg/kg i.p.) was injected again 45 min after apomorphine, whereas injection of haloperidol (1mg/kg i.p.) during the first 30 min of the intense apomorphine effect did not stop the turning or induce catalepsy in either MR- or DR-lesioned rats.

It should be noted that, although DR-lesioned animals tended to turn in response to apomorphine following chronic haloperidol pretreatment, the turning rate was just over the arbitrarily set limit of 30 turns/30min. In contrast to the MR-lesioned rats, not an increase but a statistically significant decrease of the turning rate was recorded 1 hour after the 15th haloperidol injection, compared to the turning following a single apomorphine injection ($p < 0.05$, paired t test) (Fig. 5.15). It should also be noted that all the other behavioural symptoms of apomorphine administration were intensified in MR- and DR-lesioned rats treated chronically with haloperidol. The behavioural effects of amphetamine (apart from circling) were also more intense when it was administered 1 hour or 24 hours after the last in a series of 15 daily haloperidol injections, than when it was administered to the same animals without this pretreatment. Hyperactivity, exploration, stereotypy (especially padding of the forepaws), piloerection, tail erection, backwards or sidewards movements and jumping were the common symptoms observed. The stereotypy was intensified after about 45 min when the turning stopped, and continued for about 1 hour with increased sidewards jumping. Sedation and catalepsy followed the disappearance of the effects of amphetamine.

5.3.4g Interaction of drugs acting on the dopaminergic and the serotonergic systems

Haloperidol (1mg/kg i.p.) administered 1 hour before the 5-HT receptor agonist 5-MDT, (10mg/kg i.p.) blocked the turning induced by

the latter, in both MR- and DR-lesioned animals. The catalepsy that resulted from the haloperidol administration was followed and replaced by the symptoms of 5-MDT, i.e. flattening and extension of the body, tremor, convulsions, squeaking (when touched), shivering, padding of the forepaws and extension of the hindpaws. Similarly, haloperidol pre-treatment abolished the turning but not the other behavioural effects of the combination phenelzine plus L-tryptophan.

Simultaneous administration of apomorphine ($2mg/_{kg}$ i.p.) and 5-MDT ($10mg/_{kg}$ i.p.) resulted in no consistent or measurable turning of the rats in either direction (Fig. 5.16.). The same response was obtained from rats lesioned in the MR or the DR, in contrast to the intense turning (but in opposite directions) induced by both agents when they were administered alone (Fig. 5.12). The behavioural effects of the 5-HT agonist were the predominant ones; the flattened body of the animal was moving with difficulty, with tremor, shivering and even convulsions dominating its posture, but with intermittent clinging on the walls of the bowl and jumping. Characteristic movement of the head to the left and to the right, with repetitive padding of the forepaws were observed.

Simultaneous administration of amphetamine ($5mg/_{kg}$ i.p.) and 5-MDT ($10mg/_{kg}$ i.p.) resulted in turning that was significantly more intense ($p < 0.025$, paired t test) in both MR- and DR-lesioned rats compared to the turning induced by amphetamine alone. The direction of turning was determined by amphetamine, i.e. rats with a lesion in the MR turned contralaterally (away from the lesioned side) and rats with a lesion in the MR turned ipsilaterally (towards the lesioned side), (Fig. 5.16). The behavioural response to this drug combination was a mixture of the effects of the two drugs. Hyperactivity and stereo-

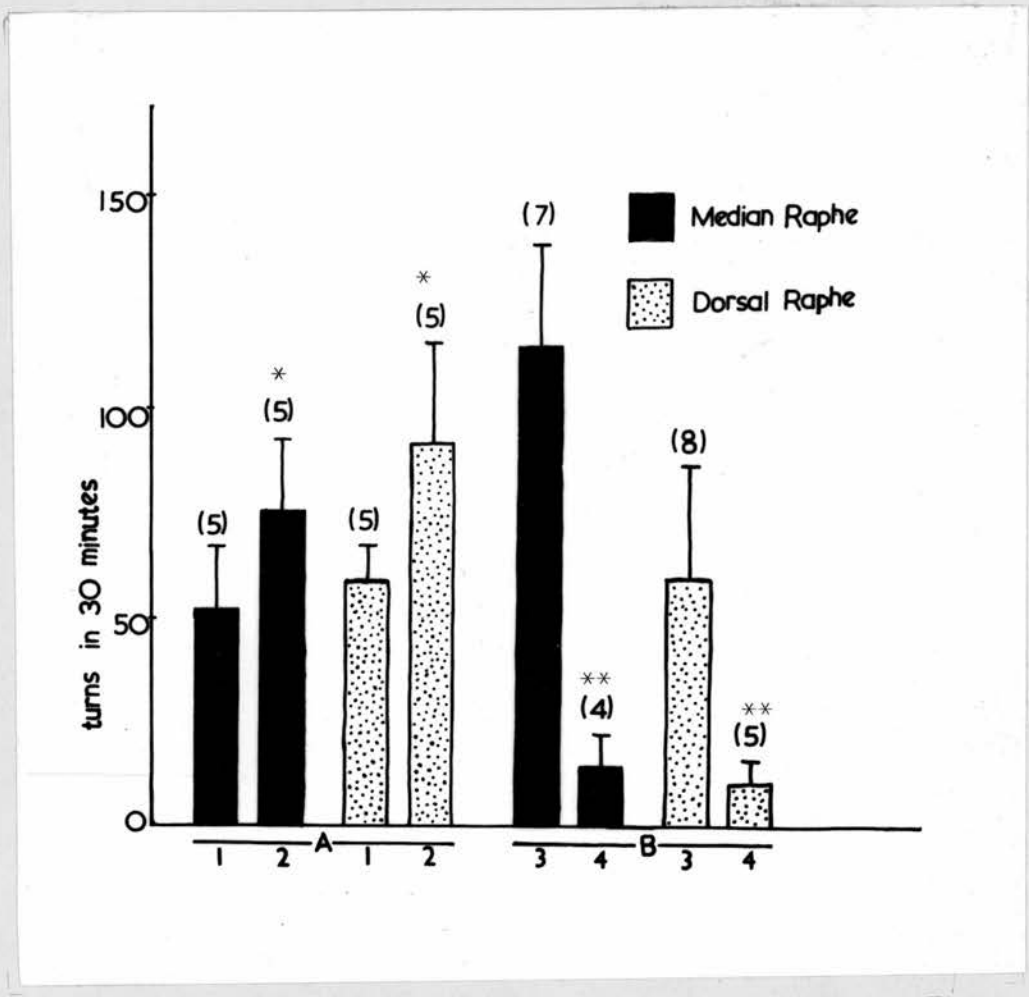


Fig. 5.16

Turning behaviour of rats with MR or DR lesions

Bars represent s.d., with the number of rats used in parentheses.

A: 1 - 5 mg/kg amphetamine i.p.
   2 - 5 mg/kg amphetamine plus 10 mg/kg 5-methoxy-N, N-dimethyltryptamine i.p.
B: 3 - 2 mg/kg apomorphine i.p.
   4 - 2 mg/kg apomorphine plus 10 mg/kg 5-methoxy-N, N-dimethyltryptamine i.p.

* $p < 0.025$
** $p < 0.0001$
(paired t test).

typed movements were coupled with body flattening and difficulty in moving, also tremor, shivering and convulsions, squeaking and hiding of the hindpaws under the body. Turning continued over 45 min.

Methysergide bimaleate, a 5-HT receptor antagonist (339), at a dose of 0.5mg/kg i.p. caused no turning in DR-lesioned rats, but induced contralateral turning in rats lesioned asymmetrically in the MR (Fig. 5.19). When injected it rendered the animals sedated but very excitable.

A drug combination which proved interesting was the administration of methysergide bimaleate (0.5mg/kg i.p.) followed 45 min later by apomorphine (2mg/kg i.p.). In all rats with an asymmetric lesion either in the MR or the DR, the behavioural effects of apomorphine, including circling, were apparent before the end of the first min after the injection and frequently as soon as 30 sec. Hyperactivity and stereotypy (sniffing, licking, clinging on the walls of the bath, etc.,) appeared in less than 1 min , in contrast to the effects appearing in about 3 min after the injection when apomorphine was given alone (without pretreatment with methysergide). The intensity of turning, measured in turns/30 min , was significantly higher after this drug combination, compared to apomorphine administration alone, both in MR- and DR-lesioned rats ($p < 0.05$, paired t test) (Fig. 5.17). The net number of turns was also significantly higher during the first 5 and 10 min in both MR and DR lesioned rats, compared to the appropriate controls ($p < 0.05$, paired t test), as shown in Fig. 5.18. The direction of circling after apomorphine was not altered by the pre-treatment with methysergide, i.e. it was contraversive in MR- and ipsiversive in DR-lesioned rats.

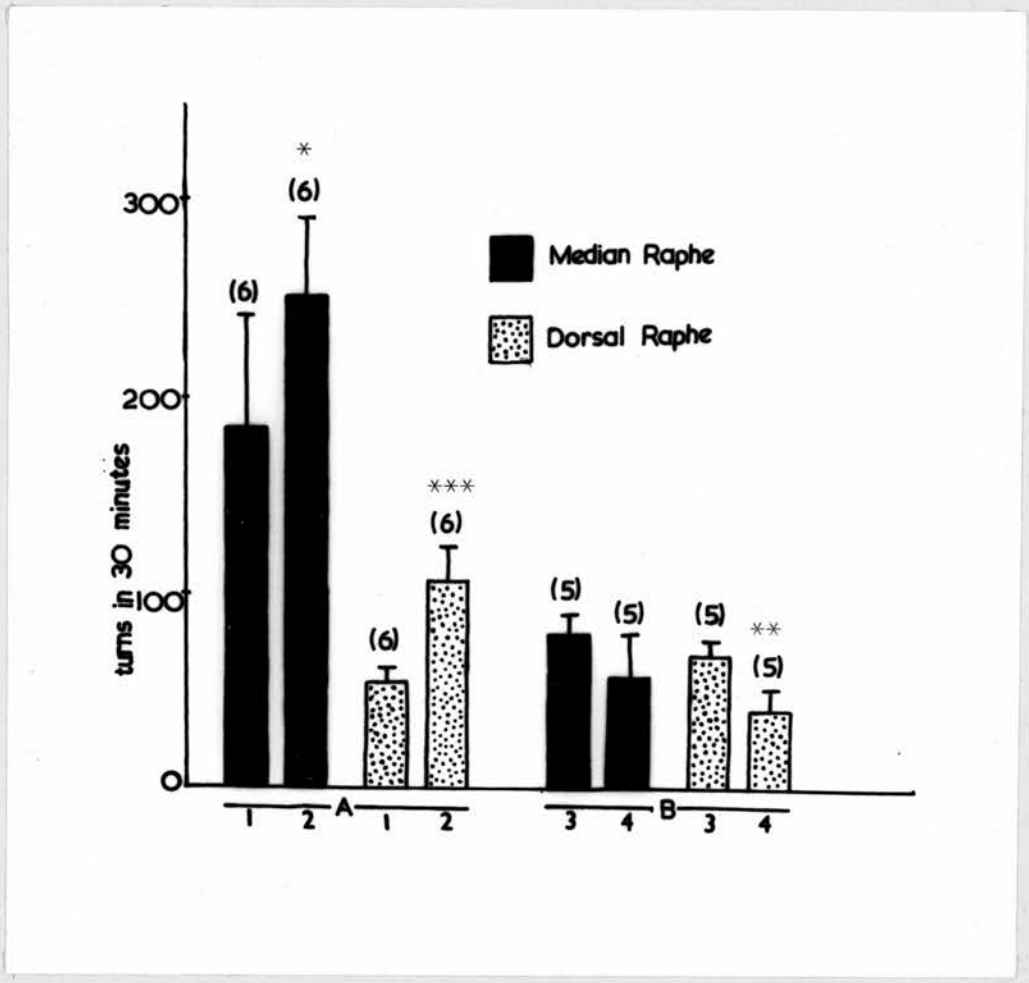


Fig. 5.17

Effect of acute administration of methysergide on apomorphine- and amphetamine-induced turning.

Vertical bars represent s.d., with the number of rats used in parentheses.

A: 1 - 2 mg/kg apomorphine i.p.
   2 - 0.5 mg/kg methysergide plus 2 mg/kg apomorphine i.p.
B: 3 - 5 mg/kg amphetamine i.p.
   4 - 0.5 mg/kg methysergide plus 5 mg/kg amphetamine i.p.

* $p < 0.05$
** $p < 0.025$
*** $p < 0.0125$
(paired t test)

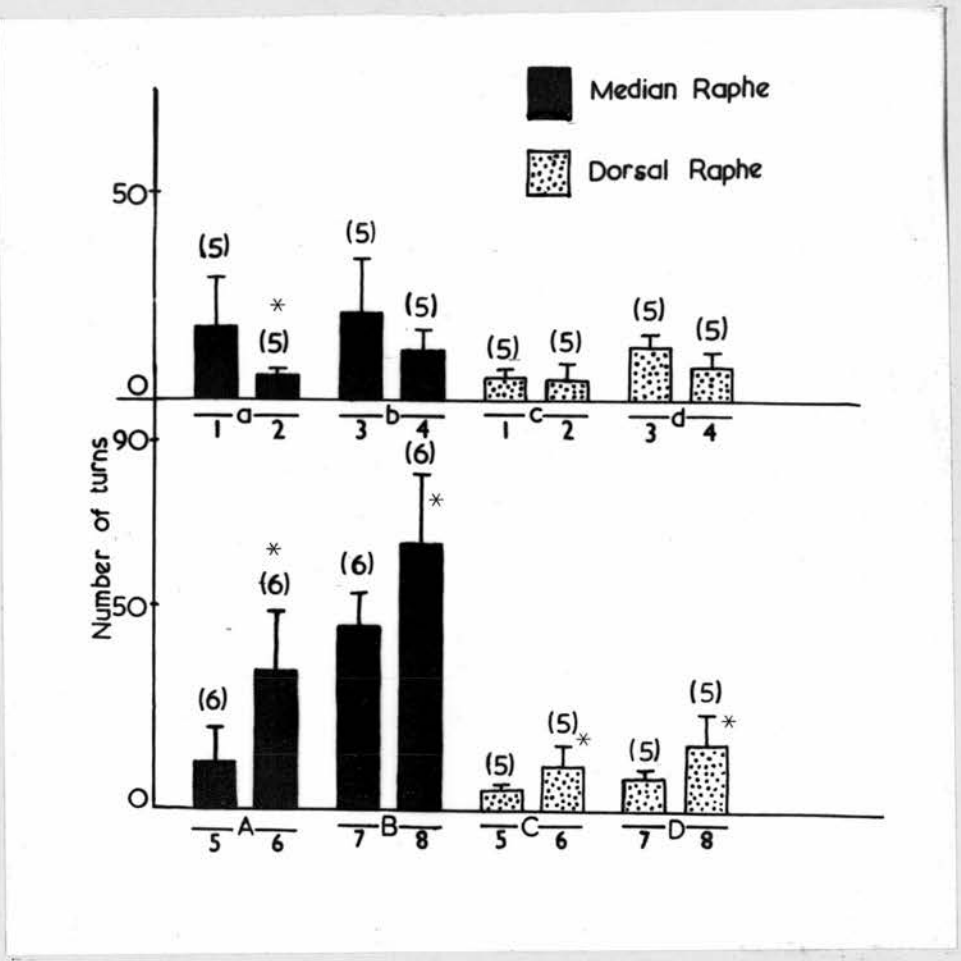


Fig. 5.18

Effect of acute administration of methysergide on the turning during the first 5 or 10 min after apomorphine or amphetamine.

Vertical bars represent s.d., with the number of rats used shown in parentheses.

a,c (5 min): 1 - 5 mg/kg amphetamine i.p.
2 - 0.5 mg/kg methysergide plus 5 mg/kg amphetamine i.p.

b,d(10 min): 3 - 5 mg/kg amphetamine i.p.
4 - 0.5 mg/kg methysergide plus 5 mg/kg amphetamine i.p.

A,C (5 min): 5 - 2 mg/kg apomorphine i.p.
6 - 0.5 mg/kg methysergide plus 2 mg/kg apomorphine i.p.

B,D(10 min): 7 - 2 mg/kg apomorphine i.p.
8 - 0.5 mg/kg methysergide plus 2 mg/kg apomorphine i.p.

* $p < 0.05$ (paired t test)

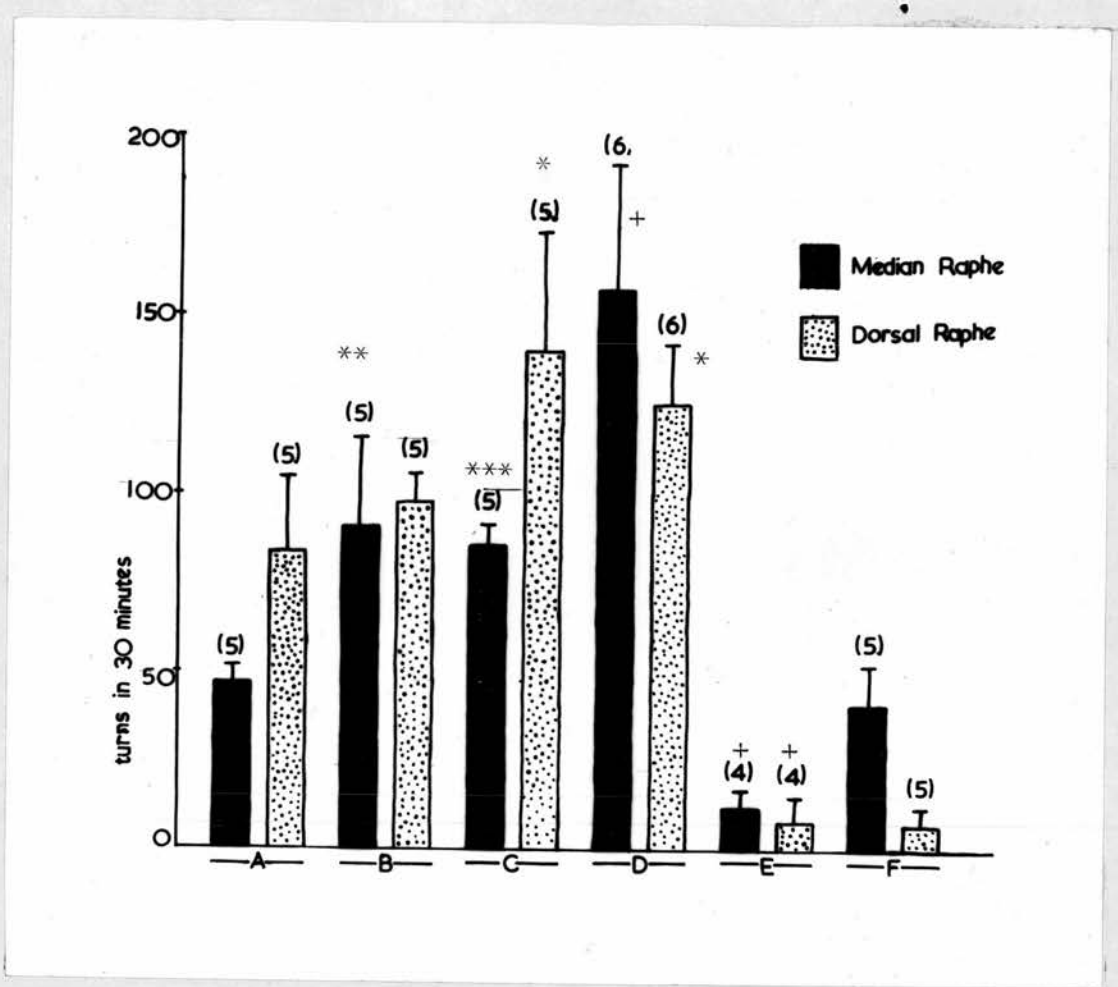


Fig. 5.19

Effect of chronic treatment of rats with methysergide on the turning induced by 5-methoxy-N,N-dimethyltryptamine (5-MDT) given 45 min after the last injection of methysergide.

Vertical bars represent s.d.; the number of rats used is in parentheses.

A - 10 mg/kg 5-MDT i.p.
B - 0.5 mg/kg methysergide (daily for 22 days) plus 10 mg/kg 5-MDT i.p. (45 min after last injection of methysergide)
C - 0.5 mg/kg methysergide (daily for 24 days) plus 10 mg/kg 5-MDT
D - 0.5 mg/kg methysergide (daily for 27 days) plus 10 mg/kd 5-MDT
E - 0.5 mg methysergide (acute) plus 10 mg/kg 5-MDT i.p.
F - 0.5 mg methysergide

* $p < 0.05$
** $p < 0.01$
*** $p < 0.005$
\+ $p < 0.0005$
(compared to A)

That the dose 0.5mg/kg i.p. of methysergide was sufficient to directly block some central 5-HT actions was demonstrated by the complete inhibition of the turning induced by the central 5-HT receptor stimulant 5-MDT (10mg/kg i.p.) when the blocker was administered 45 min before the agonist drug. It was observed that most of the behavioural effects of the 5-HT agonist were apparent despite the blockade of 5-HT receptors. These behavioural effects could not, possibly, be inhibited by doses of 0.5 or 1mg/kg i.p. of the antagonist, which, however, were effective in inhibiting circling completely. Thus, the animals were sedated after methysergide (or were slowly turning in the case of MR lesions) but showed a burst of activity immediately after the injection of the 5-HT agonist, flattening of the body within about 2 min , shivering,tremor and convulsions, padding of the forepaws and intermittent clinging on the walls of the bath.

Administration of methysergide (0.5mg/kg i.p.) followed 45min later by DL-amphetamine (5mg/kg i.p.) caused turning in the same direction as with DL-amphetamine alone, (i.e. contralateral in MR and ipsilateral in DR lesions), but of lower intensity (measured as turns/ 30min) (Fig. 5.17). The intensity of turning in MR-lesioned rats was significantly lower during the first 5 min ($p < 0.05$, paired t test) (Fig. 5.18), but not during the 10 or the 30 min of testing, compared to a single administration of amphetamine. There was no significant effect on the turning rate of DR-lesioned animals during the first 5 or 10 min (Fig. 5.18) but a significant reduction during the 30 min period (Fig. 5.17). The turning started in less than 60 sec after the amphetamine injection and continued for up to 90 min. Hyperactivity, exploration and stereotyped movements were not marked during the first 20 min , but thereafter increased in the period of

20–40 min , reaching the maximum at 40–45 min.

5.3.4h Tolerance to chronic treatment with methysergide

As described above and shown in Fig. 5.19, an acute dose of 0.5mg/kg i.p. of methysergide bimaleate inhibited the turning induced by 10mg/kg i.p. of 5-MDT when the latter was injected 45 min later. When the 5-HT receptor blocker, however, was administered daily at the dose of 0.5 mg/kg i.p. for 22 days to animals with a lesion in the MR or the DR, an injection of the 5-HT receptor agonist at a dose of 10mg/kg i.p. 45 min after the last injection of the inhibitor, initiated intense turning. The direction of turning induced by 5-MDT was the same as that recorded in the absence of any chronic pretreatment with methysergide, i.e. ipsilateral to the lesioned MR side and contralateral to the lesioned DR side. The intensity of turning was higher in both MR and DR lesion and even reached the level of statistical significance in rats with a lesion in the left side of the MR ($p < 0.01$, paired t test), as Fig. 5.19 illustrates. As the treatment with methysergide continued, daily, for 24 or 27 days, the rats of both MR and DR lesion type responded to a challenge with 5-MDT (10mg/kg i.p.) with a further increase in intensity of turning, which reached statistical significance in both groups of animals (Fig. 5.19). An increase in the last dose of methysergide by two or four times (i.e. 1mg/kg or 2mg/kg) did not inhibit the turning initiated by a single dose of the 5-HT receptor stimulant (10mg/kg i.p.) given 45 min later. A decrease in the intensity but not complete elimination of turning could be obtained when, in addition to the last (27th) injection of 0.5mg/kg i.p. of methysergide, 4mg/kg i.p. of the latter agent were injected into the rats at the same time as 5-MDT. The turning rates obtained were

significantly lower (about 50%) than in the absence of the additional 4mg/kg of methysergide, in both MR- and DR-lesioned rats ($p < 0.05$, paired t test), thus becoming approximately equal to the rates of turning after a single injection of 5-MDT.

Chronic administration of methysergide for 15 days, at a daily dose of 0.5mg/kg i.p., followed on the last day by apomorphine (2mg/kg i.p.) 45 min after the last methysergide injection, resulted in no modification of the acute effect of this drug combination regarding the intensity and direction of turning (Fig. 5.17). The turning rates recorded were significantly higher compared to those of apomorphine alone in both MR- and DR-lesioned rats ($p < 0.05$, paired t test). There was no change in the direction of turning and no statistically significant difference in the intensity of turning after this drug combination, either when methysergide was administered once (45 min before apomorphine) or when it was administered for 15 days (with the last injection 45 min before apomorphine).

5.3.4i Amphetamine-induced turning: effects of drugs

Pretreatment of rats with haloperidol (1mg/kg i.p.) for 1 hour prevented the amphetamine-initiated turning, as has been described above, in both MR and DR type of lesion.

Propranolol, a centrally acting β-adrenergic receptor blocker (187), which recent reports indicate is active in central serotonergic mechanisms (371) was administered at a dose of 10mg/kg i.p. (effective in blocking the central noradrenergic system) (187) 90 min prior to the administration of amphetamine (5mg/kg i.p.) and the turning which resulted was compared to that recorded when amphetamine alone was given. No change in the direction of turning was observed and no statistically significant difference in the intensity of turning was recorded, either

in MR- or DR-lesioned rats (Fig. 5.20). Similarly, injection of phenoxybenzamine (a central α-adrenergic receptor blocker)(372) at a dose of 30mg/kg i.p. failed to alter the intensity or the direction of the circling response of rats with MR or DR lesion to amphetamine, 5mg/kg i.p.

Furthermore, fusaric acid, a drug thought to act primarily by inhibiting the enzyme which converts DA to NA (that is dopamine-β-hydroxylase) and hence decreases the central NA concentration (373) was combined with amphetamine and the effect of this drug combination was compared to the effect of amphetamine on circling behaviour. Although amphetamine alone (5mg/kg i.p.) induced circling that was of relatively higher intensity than the circling induced by the combination of this drug with fusaric acid (75mg/kg i.p.) given 90 min prior to its administration, no statistically significant difference was found between the two treatments in either MR or DR lesions (Fig. 5.20).

With regard to the other behavioural effects, propranolol caused deep sedation of the animals, which was followed by hyperactivity, exploration and stereotypy, clinging to the walls, uplifting of the forelimbs and exploration of the air space, piloerection, etc., when amphetamine was injected. The circling persisted for about 60-70 min. Administration of fusaric acid also induced deep sedation followed by the effects of amphetamine, such as hyperactivity, exploration and stereotypy.

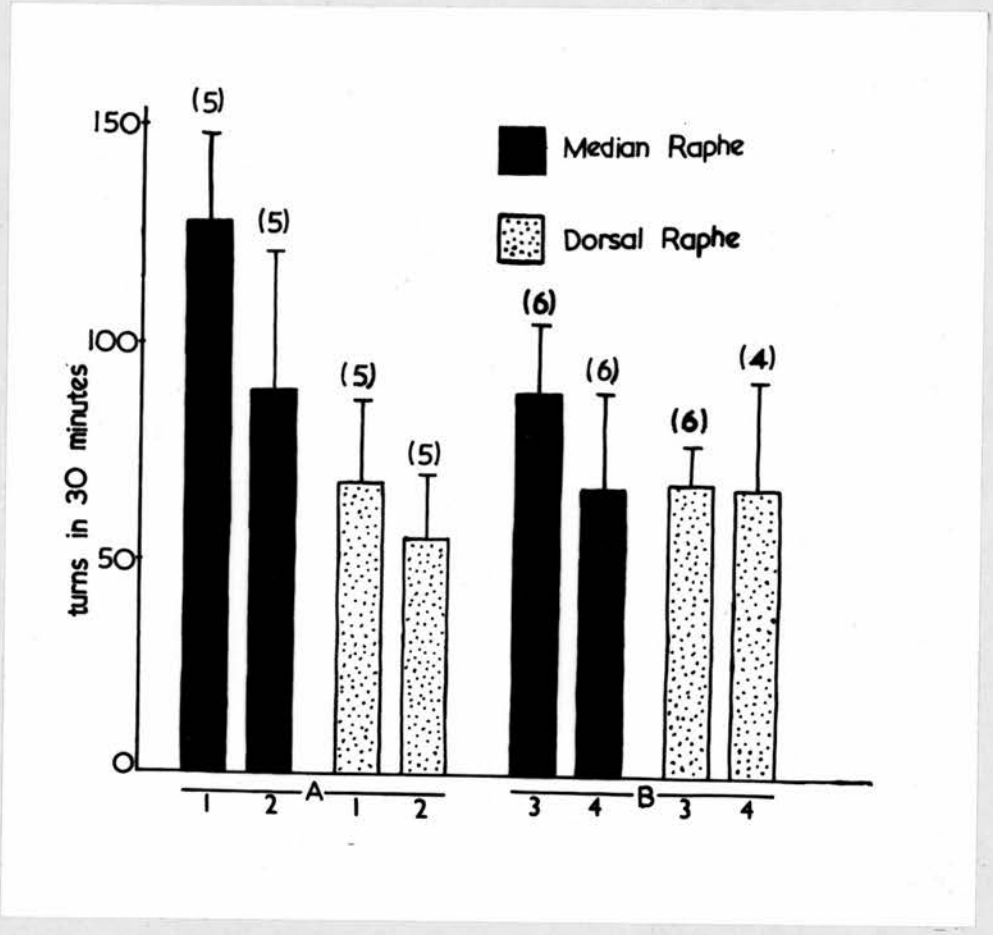


Fig. 5.20

Effect of fusaric acid and propranolol on the turning induced by amphetamine

Vertical bars represent s.d., with the number of animals used in parentheses.

A: 1 - 5 mg/kg amphetamine i.p.
   2 - 75 mg/kg fusaric acid plus 5 mg/kg amphetamine i.p.
B: 3 - 5 mg/kg amphetamine i.p.
   4 - 10 mg/kg propranolol plus 5 mg/kg amphetamine i.p.

5.4 DISCUSSION

5.4.1 Anatomical significance of the results

It is well established that when the cell bodies of central neurons are destroyed, the axons and the nerve terminals of these neurons degenerate within a few weeks, with a concomitant disappearance from the innervated areas, of the neurotransmitter substance utilised by them. On the basis of this fact, lesioning of specific raphe nuclei was performed and the brain areas thought to be innervated by projections from these nuclei were examined for changes in 5-HT, the neurotransmitter believed to be utilised by these projections. Since the functional state of an aminergic neuronal system is related to the metabolism of its neurotransmitter (25, 26,27), an assessment of the effects of lesions of the serotonergic nerve cell bodies on the functional activity of the 5-HT neurons could be primarily assisted with measurement of the concentrations of 5-HT and its main metabolite, 5-HIAA, in certain brain structures where the suspected serotonergic innervation terminates. Such areas are the corpus striatum and the substantia nigra of the rat brain, which, as mentioned in the Introduction, are believed to receive serotonergic projections from the raphe area, and where 5-HT may act as neurotransmitter.

The 5-HT content of the terminal area could be used as an index of a successful lesion at the cell bodies of serotonergic neurons innervating it. Therefore, the 5-HT levels, as well as the levels of its main metabolite 5-HIAA, were measured in each side of corpus striatum and substantia nigra after selective lesions of the raphe cell

groups B7 (dorsal raphe, DR) and B8 (median raphe, MR). The lesions were placed unilaterally, i.e. in one side of the raphe nucleus and the left and right sides of the two suspected terminal areas were separately dissected and analysed. This procedure was based on the assumption that there is no important crossing of raphe-originating ascending serotonergic fibres to the contralateral side of the brain. To our knowledge there is no clear-cut anatomical information in the literature about this point, although this was implied by the histochemical fluorescence-based mapping of brain monoamine pathways by Ungerstedt (8) and by Fuxe and Jonsson (301). It was reasonable to conclude that this was the case with regard to the striatal and nigral innervation, since asymmetric lesions of the raphe nuclei had an effect (if any) on only the 5-HT and 5-HIAA levels of the ipsilateral side and not the contralateral side.

2-3 months after the asymmetric lesions, 5-HT and 5-HIAA were reduced to about the same extent, i.e. about 30 per cent, in the striatum ipsilateral to the MR lesion and in the substantia nigra ipsilateral to the DR lesion. Lesions of the DR had no significant effect on striatal 5-HT, and lesions of the MR did not alter the nigral 5-HT. These differential neurochemical effects of lesions of the two raphe nuclei were supported by the histological assessment of the lesion location and the distinct behavioural responses of the lesioned animals. In addition, other biochemical results supported this finding: lesions in the left side of MR had only an effect on DA metabolite concentrations (HVA and DOPAC) in the left side of the striatum but not in the substantia nigra, whereas lesions in the left side of DR had an effect on HVA and DOPAC in the left substantia nigra but not in the striatum. Since it is known that there is no significant

crossing of the DAergic fibres of the nigrostriatal pathway (8), the effects of the unilateral lesion on DA metabolism may be seen only unilaterally.

Treatment of lesioned animals with a large dose of L-tryptophan (100mg/kg i.p.), which is known to increase the 5-HT synthesis in the brain (Section 4) did not in general alter the pattern of changes. The predominantly differential projections of the two raphe nuclei were again confirmed, but with an additional important point: lesions of the DR resulted in a smaller increase in 5-HT and 5-HIAA (which reached statistical significance) in the ipsilateral compared to the contralateral side of the striatum, contrary to the lack of any difference between the two sides in saline-treated rats. In addition, a much smaller increase of 5-HT and 5-HIAA was found in the ipsilateral substantia nigra compared to the contralateral side ($p < 0.01$, paired t test), so that the concentrations of 5-HT and 5-HIAA in the ipsilateral side were 45% and 74%, respectively, of the contralateral side.

Two main points arise from these findings. Contrary to various previous reports, these data suggest that the MR is the main origin of the striatal serotonergic innervation and DR the main origin of the serotonergic innervation of the substantia nigra. With regard to the origin of the serotonergic afferents to the substantia nigra, the results of the present study are in agreement with the finding of Bunney and Aghajanian (305), who demonstrated by the use of the retrograde horseradish peroxidase technique, that the origin of these fibres is the DR and not the MR; they are, however, contrary to the results of the work of Dray et al. (87) which support the MR and not the DR as the origin of the nigral serotonergic innervation.

The evidence of Dray et al.(87) is weakened by the fact that they approached the MR area vertically, possibly thereby damaging the DR nucleus with the electrode; this casts some doubt on the specificity of the lesions and the electrophysiological experiments they performed. Furthermore, the fact that they performed large lesions of the MR nucleus, which probably damaged other raphe cell groups and even the DR nucleus (mainly by secondary gliosis and cell degeneration) could be another reason for the different results of their study, compared to the well localised, small lesions of the present study.

It seems difficult to explain the difference of the present results from several previously published reports regarding the striatal serotonergic afferent. The exclusiveness of the MR cell group as the origin of this projection is indicated by the evidence from the present experiments, although the tryptophan loading revealed that some, perhaps limited, serotonergic innervation of the striatum may originate from the DR. This disagrees, however, with the results of several lesion studies (302,309) suggesting that the DR is the major origin of the 5-HT projections to the striatum, or with the evidence of experiments using the labelled amino-acid transport method (310) or the horseradish peroxidase technique (302,311), which point to the same conclusion. In support of the present study, on the other hand, is the work of Costall and Naylor (314). They made small asymmetric electrolytic lesions of the DR and the MR, confined to part of these cell groups (a methodology followed in the present study) and obtained biochemical evidence suggesting that the MR and not the DR sends serotonergic fibres to the striatum. The results of Marsden and Guldberg (313) based on lesion studies, also give support to the finding that the MR is the major origin of the serotonergic

innervation of the striatum, as well as of the cerebral cortex and the hippocampus. They demonstrated, however, that large lesions which destroyed both the MR and the DR resulted in a bigger decrease of striatal 5-HT compared to large MR lesions, indicating that the DR or other nuclei located between MR and DR may also project to the striatum. A common feature of the studies carried out in the past (302,297) that involved lesions of the raphe nuclei, seems to be the extensive damage of the whole nucleus and also some of the adjacent areas; in the case of DR lesions, for example, the damage frequently extended as far as the area pedunculus cerebellaris superior, (Fig. 5.1), where 5-HT cells also seem to exist (5). In the present experiments it was observed that lesions affecting these areas did not produce the same consistent pattern of behavioural response (circling) after amphetamine or apomorphine, which was produced by small lesions in a restricted area of the DR. Therefore, these animals were discarded from further experiments. This point could be a methodological reason for the different results, if indeed the 5-HT cell bodies adjacent to the DR send projections to the striatum.

It is also possible that, some of the cell bodies of the raphe nuclei thought to be destroyed by the lesion contain another indolealkylamine, e.g. tryptamine (as it has been suggested by other investigators, 369,301); damage to these cell bodies by one lesion (present study) and not by others (previous work), and vice-versa, could account for the different results of lesions seemingly affecting the same brain area. Although the nature and the physiological role of the raphe cells which contain indolealkylamine(s) other than 5-HT is not known, the interpretations of the electrophysiological experiments (302,312) could be seriously weakened if these cells had

a different function and different response upon electrical stimulation than the 5-HT containing cells. Autoradiographic studies based on the retrograde transport of labelled aminoacids, which indicate that the DR nucleus innervates the striatum (302,311,310) could also suffer from the reported limitations of the technique: non-specific damage and diffusion, uptake of the labelled tracer by axons on passage, orthodromic and antidromic transport of the lebelled material, etc. (370). Similarly, electrophysiological experiments could be misled by current spread to dorsal raphe axons during the recording. Therefore, in the raphe area where interconnections of the various nuclei seem to exist (325,327), misleading results could be obtained using these techniques in order to identify projections originating from a particular cell body area.

The discrepancy between saline-treated and tryptophan-treated rats concerning the existence of a serotonergic projection from the DR nucleus to the striatum may be important. The possibility that this difference may be due to methodological difficulties also has to be borne in mind. The employed method of Curzon and Green (288) is sensitive for the measurement of 5-HT and 5-HIAA in a single striatum in saline-treated rats, but it may not be sufficiently discriminating subtle changes of the concentrations within the range 10-20%. Tryptophan loading increases significantly the concentrations of 5-HT and 5-HIAA, and this might improve the accuracy of the assay to the extent that changes otherwise not revealed become apparent. Therefore it is possible that a minor serotonergic innervation of the striatum originates from the DR nucleus, as the difference between the two sides of the striatum after tryptophan loading indicates. Otherwise, a preferential effect of the lesion on the synthetic enzymes of

5-HT could also be the reason for the discrepancy: when synthesis of 5-HT is increased by the administration of tryptophan, the difference in the activity of 5-HT neurons between the denervated and the intact side of the striatum may be amplified, resulting in a bigger difference in the concentrations of the amine and of its metabolite between the two sides.

Tryptophan loading, either alone or in combination with MAO inhibition, is known to increase to a different degree both 5-HT and 5-HIAA concentrations in the rat brain (Section 4). In the case of the raphe lesions, a treatment that increases the synthesis and metabolism of 5-HT to a different but known extent (such as tryptophan loading) would be a useful tool in differentiating the metabolic stages affected by the denervation of the brain structure under study. It would also make the determination of 5-HT and 5-HIAA in small brain areas, such as substantia nigra, possible without the need to pool together 2 or 3 tissue pieces from different animals.

The demonstrated significant reduction of the ratio 5-HT/5-HIAA in the substantia nigra ipsilateral to the DR lesion compared to the contralateral side, following the administration of a large dose of L-tryptophan, may indicate a deficit in 5-HT synthesis or an increase in 5-HT metabolism caused by the lesion. A preferential, bigger reduction of uptake of precursor (tryptophan or 5-HTP) by the serotonergic terminals in the denervated substantia nigra, or a reduction of tryptophan hydroxylase, the rate-limiting step in 5-HT synthesis, or an increase in 5-HT metabolism could be responsible for the observed deficit. The presented evidence favours an effect on 5-HT metabolism rather than on synthesis; the relatively higher increase of 5-HIAA in the denervated substantia nigra may result from increased

release of 5-HT due to destruction of storage sites in serotonergic nerve terminals.

Differences between the weight increase of rats with lesions in the MR or the DR and the sham-operated controls were recorded and were more profound during the period of 2-3 months after the lesion (Fig. 5.2). An even significant decrease of the weight of MR-lesioned rats was found, 90 days after the lesion, compared to sham-lesioned animals. These differences may be related to the observed biochemical deficits of the denervated side of the substantia nigra following tryptophan loading of DR-lesioned rats, since the animals for biochemical analyses were killed during this period. A change in the weight could be of importance for the interpretation of changes in 5-HT metabolism, since 5-HT availability and synthesis from its precursor tryptophan is subject to dietary variation, as indicated by the work of Milson and Pycock (374). These workers also demonstrated a significant alteration of the spontaneous locomotor activity and the amphetamine-induced circling behaviour in mice with nigro-striatal dopaminergic nerve terminal destruction by varying the protein content in the diet of the animals. Therefore, changes in the weight of the rats could result from changes in the dietary intake, which could also affect the tryptophan and 5-HT concentrations in the brain, and possibly to a different degree between the intact and the denervated side.

5.4.2 Evidence for functional interaction of 5-HT with dopamine

The examination of changes in amine metabolism induced in one system by selectively manipulating another system provides some information about their functional interaction in the brain. Therefore, the assessment of any changes in catecholamine metabolism in specific brain areas after lesions of the serotonergic nerve cell

bodies in the MR or DR would be of interest, especially in view of the anatomical, biochemical and pharmacological-physiological evidence for an interaction between these monoamines. The estimation of the catecholamine concentrations, namely dopamine and noradrenaline, in the striatum was of particular importance in order to establish the neuronal system responsible for the circling behaviour of rats with lesions in the MR or the DR. Some of the rats had the lesion in the area between the MR or DR and the adjacent lateral reticular formation, where ascending catecholaminergic fibres exist (5,8). Therefore, the possibility had to be examined that damage to these fibres was a contributing or even the main factor causing the turning of the animals in response to various agents acting directly or indirectly on catecholamine receptors. As the histological assessment of the lesion location could not answer this question completely, the concentrations of NA and DA in the striatum were determined. No statistically significant difference in the concentration of either of the two amines was found between the two sides of the striatum, after MR or DR lesions.

Rats with unilateral electrolytic lesions of the nucleus locus coeruleus, known to contain the bulk of the noradrenergic cell bodies in brain (5), turned in tight circles away from the side of the lesion after the administration of amphetamine or apomorphine (375). This circling in response to drugs was short lasting and usually disappeared within about 30 days of surgery. Ascending dorsal noradrenergic fibres from the locus coeruleus, located in close proximity to the MR nucleus (in the area reticular formation) could be damaged by the asymmetric lesions of this nucleus, and this could possibly result in the transient circling behaviour as it has been shown by other workers

(368). The direction of turning induced by apomorphine and amphetamine in rats with asymmetric electrolytic lesions of the MR or the locus coeruleus was found to be the same (368,378), but the turning was transient in the case of locus coeruleus lesions. Selective lesions of one side of the MR (verified histologically) not affecting the reticular formation area, were found by these workers to produce persistent, contralateral turning following the administration of DA receptor agonists (apomorphine, amphetamine); the concentration of 5-HT in the ipsilateral cortex, the limbic area and the striatum was reduced, but no effect was found on DA or NA concentrations in these areas (314,313).

The present study confirmed the above. As shown histologically, the area damaged by the lesion was small and confined to the MR or DR areas in rats successfully rotating after apomorphine or amphetamine. The concentrations of DA and NA in the ipsi- or contralateral side of the striatum were not affected by either of the two lesions. These findings indicate that the lesions did not damage any ascending DAergic or NAergic fibres and that the 5-HT system was selectively damaged. Therefore, any changes in the metabolism of DA or NA in the terminal areas could result from an indirect effect of the lesion, through synaptic contacts of the 5-HT system (which is primarily affected) with these amine systems.

However, functional variations are better indicated by changes of amine metabolism and/or turnover rather than by changes in the concentrations of the amines themselves. The fact that DA or NA concentrations did not seem to change in the striatum after raphe lesions, does not exclude the possibility of a functional link between DA or NA and 5-HT in the striatum or elsewhere. Although the assays

of the two catecholamines were performed 2-3 months after surgery, when the degeneration of the lesioned neurons should have been completed, and the resulting depletion of the amines should become obvious, the possibility exists that compensatory overactivity of the remaining intact neurons may replace the amine loss. This, however, does not seem to happen, as the changes in the metabolite concentrations indicate: The HVA and DOPAC concentrations were selectively increased in the side of the substantia nigra ipsilateral to the DR lesion or in the side of the corpus striatum ipsilateral to the MR lesion reflecting increased DA neuronal activity. Thus the data in Tables 5.1 and 5.3 demonstrate reciprocal changes in 5-HT and 5-HIAA on one hand, and in HVA and DOPAC on the other hand in these brain areas. The fact that the concentration of DA in the ipsilateral striatum did not change after MR lesions, but the concentrations of the DA metabolites increased significantly, indicates an increase in the turnover of this amine in the denervated structure. Although the DA concentration in the substantia nigra was not measured, because of the low sensitivity of the method, the selective increase in HVA and DOPAC in the substantia nigra ipsilateral to the DR lesion indicates that probably a similar release or turnover effect occurs in this area. It seems, therefore, that selective reduction of 5-HT in the striatum or the substantia nigra is accompanied by an increase in the turnover of DA in these brain structures.

Since the evidence from these experiments suggests that there is no direct effect on DAergic neurons, the lesion seems to release an inhibitory effect of the 5-HT neurons in the striatum and the substantia nigra upon the turnover of DA in these areas. That a tonic inhibition of DAergic neurons in the striatum is the normal function of 5-HT

released from afferents to this structure, has been shown by several investigators. Electrical stimulation of the DR and MR cell groups has been found to result in release of 5-HT in the striatum (283) and marked inhibition of striatal neurons (312). Microiontophoretic application of 5-HT inhibited the striatal neurons (376). Lesions in the known ascending projections from the raphe area reduced the inhibitory effect of raphe stimulation on striatal neurons (302). Similarly, microiontophoretic application of 5-HT inhibited the DA cells in the area pars compacta of the substantia nigra (315,87,64). This effect was blocked by methiothepin (64) which has been reported to be an effective antagonist of 5-HT-induced depression of cell firing in other areas of the brain (377).

A functional balance between DA and 5-HT in the striatum has been proposed by Barbeau (177) in relation to some neurological and pharmacological aspects of human parkinsonism. Several findings from animal experiments also point to a participation of 5-HT in the function of the DAergic system. The biochemical data from this study suggest that removal of the 5-HT innervation from the striatum and the substantia nigra stimulates indirectly and independently the activity of the DAergic neurons in the two structures. Therefore, a possible role of the 5-HT input in controlling the activity of DA cell bodies (in the substantia nigra) and DA nerve terminals (in the corpus striatum) might be indicated by the demonstrated inverse correlation between 5-HT concentration and DA turnover.

5.4.3 Behavioural expression of the biochemical interactions

Differential behavioural and neurochemical effects following DR or MR lesions in the rat have also been found by several other investigators. Jacobs et al.(354) have demonstrated that a total MR

lesion gives rise to increased locomotor activity, but a DR lesion has no effect on locomotor activity. Similarly, Srebro and Lorens (378) have shown that MR lesions increase responsiveness to novel stimuli or environmental change, whereas DR lesions do not produce these effects, despite the fact that MR lesions produced a decrease in forebrain 5-HT that was smaller (26%) than that produced by DR lesions (65%). According to Geyer et al. (379), it is the MR lesion, and not the lateral (DR) lesion, which is responsible for the hyperactivity and hypersensitivity that follows combined raphe lesions. The main difference in projections according to these authors (379) is that the MR projects to the septum and the hippocampus, whereas the DR does not. Both project to the hypothalamus and cortex. The MR is considered by several authors to be the primary source of hippocampal 5-HT (309,354,379). Further work of Jacobs et al. (380), however, contrary to the above, indicates that the increased locomotor activity following a DR lesion or depletion of 5-HT induced by p-chlorophenylalanine is abolished by prior aspiration of the anterodorsal hippocampus in adult rats. A common finding of these studies despite the controversy, is the increased locomotor activity of rats following selective decrease of 5-HT in brain and, probably, a differential behavioural expression of lesions in the MR or the DR.

A state of general behavioural excitation, with increased locomotor activity was observed after the administration of drugs which are known to increase the activity of catecholamine containing neurons in the brain (363), whereas drugs that reduce central catecholamine transmission produce behavioural states of sedation and inactivity. Similarly, behavioural excitation with increased locomotor activity is observed after pharmacological and neurological procedures which lower

selectively brain 5-HT, e.g. inhibition of 5-HT synthesis by p-chlorophenylalanine (319,320) or destruction of serotonergic neurons by electrolytic lesions placed in the midbrain raphe (321), preferably in the MR rather than in the DR nucleus (354,379).

In the present study, in addition to the biochemical differentiation, the behavioural response of rats with an asymmetric electrolytic lesion in the MR or the DR was different following the administration of various drugs. Most characteristic of these responses is the circling behaviour, as depicted in the diagram of Fig. 5.21. Amphetamine and apomorphine induced contraversive circling in MR lesions and ipsiversive in DR lesions. These responses were dose-dependent and could be obtained up to 4 months after the operation. Other workers have reported circling behaviour of raphe lesioned rats (321). Moreover, selective asymmetric lesions of the MR were found to result in consistent contraversive circling behaviour following the administration of various doses of apomorphine or amphetamine (368,314). There was no consistent spontaneous rotation with either of the lesions, perhaps because the survival of a relatively small number of neurons may suffice for conditions where demand is within normal limits.

Ungerstedt et al. (381) have suggested that the induction of circling behaviour after lesions of the nigrostriatal neurons is almost exclusively a DAergic phenomenon. Furthermore, Ungerstedt (8) and Creese and Iversen (382) have suggested that the amphetamine-induced locomotor and stereotyped behaviour are both dependent on the functional integrity of the nigrostriatal system. When the nigrostriatal DAergic pathway is lesioned unilaterally, administration of apomorphine induces contraversive circling, whereas amphetamine induces ipsiversive circling. These responses have been interpreted, the former

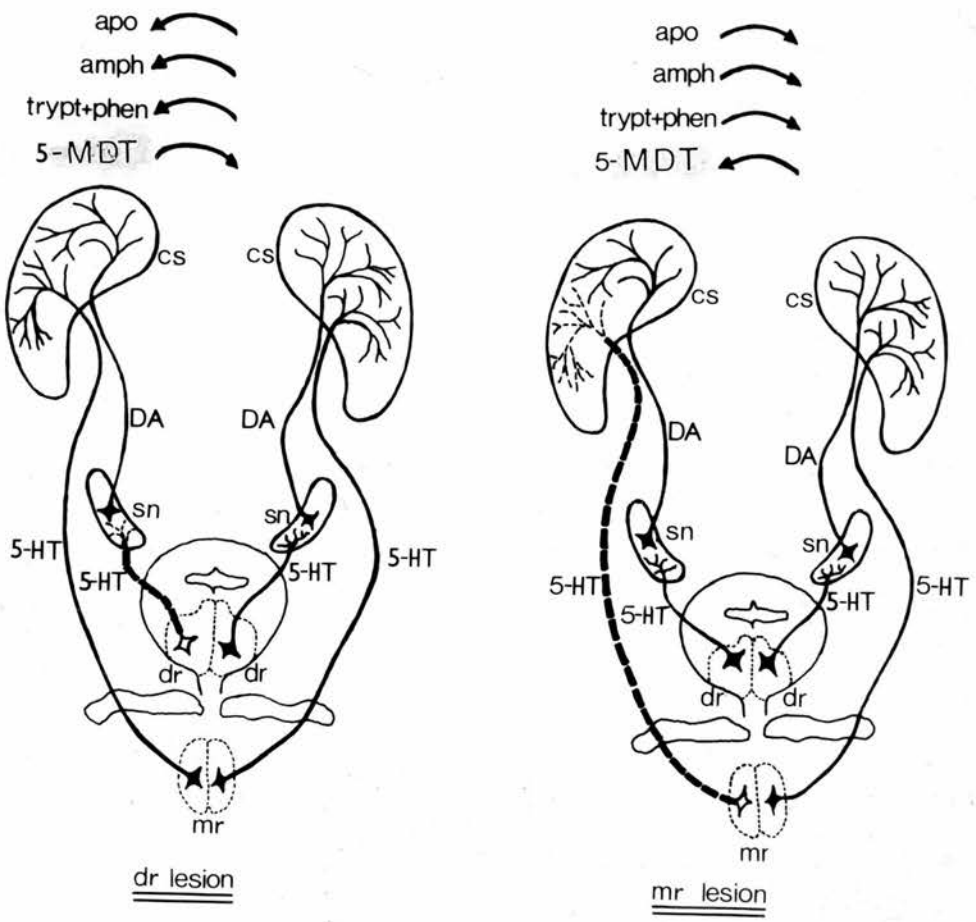


Fig. 5.21

A schematic representation of the serotonergic innervation of the substantia nigra (sn) and the corpus striatum (cs) by fibres arising unilaterally from the dorsal raphe (dr) and the median raphe (mr) respectively, as suggested by the present study. The degeneration of 5-HT axons (broken line) after asymmetric lesions of the raphe nuclei produced turning upon stimulation by various drugs in the direction indicated by the arrows. The nigrostriatal pathway is also represented.

Abbreviations: apo: apomorphine, amph: amphetamine, trypt + phen: tryptophan plus phenelzine, 5-MDT : 5-methoxy-N,N-dimethyltryptamine

as being due to stimulation of the supersensitive DA receptors in the denervated striatum, and the latter as being due to release of DA from the intact striatum (8,110). In both cases, a striatal DAergic deficit and an imbalance is involved.

In the present study, the amphetamine- and apomorphine-induced turning seem to be, partly at least, DAergic phenomena. Amphetamine is known to release both NA and DA from the presynaptic sites, but for the circling behaviour of rats lesioned in the MR or the DR, it seems that there is no NA involvement, at least at the striatal level. This was suggested by the ineffectiveness of various drugs acting on NAergic transmission to alter the rate of amphetamine-induced turning. These drugs were: a β-adrenergic receptor blocker (propranolol), an α-adrenergic receptor blocker (phenoxybenzamine) and a DA-β-hydroxylase inhibitor (fusaric acid). Doses of these agents that had been found effective in altering central NAergic transmission were used (187,372,373). Thus the amphetamine-induced circling behaviour seems to be due to receptor stimulation by the released DA. Lowering of striatal 5-HT after the asymmetric MR lesion appeared to release the striatal DAergic neurons from an inhibitory influence, as the increase in DA turnover indicates. This unilateral increase in DA-ergic transmission might have caused the spontaneous contraversive turning which was enhanced by the postsynaptic DA receptor stimulant apomorphine and the DA releasing drug amphetamine. The effects of both drugs were completely blocked by haloperidol, a DA receptor blocker, and this further supports the concept of a DAergic mechanism involved in the turning.

A similar mechanism could exist in the DA cell body area of the substantia nigra, where lowering of 5-HT (after lesions in the DR) was

followed by an increase in the concentrations of HVA and DOPAC. The nature of the 5-HT projection to the substantia nigra also seems to be inhibitory, as several previous studies indicate, in agreement with the increase in DA turnover suggested by the present experiments. Stimulation of the DA receptors, however, which are believed to exist on DAergic cells or dendrites of the substantia nigra (53) or on non-DAergic neurons (46,56), by apomorphine, or by amphetamine (indirectly) produced circling ipsiversive to the lesioned side in rats with an asymmetric lesion in the DR. Thus, removal of the inhibitory effect of 5-HT neurons from DA cell bodies or DA terminals resulted in turning in opposite directions upon stimulation with DA receptor stimulants (Fig. 5.21).

If the integrity of the nigrostriatal DAergic system is necessary for the amphetamine-induced locomotor activity and stereotyped behaviour, as it has been proposed (8,382), then an effect on the DA cell bodies in the area pars compacta of the substantia nigra should result in the appropriate response of the striatal target cells or neurons. Thus an inhibition of the DA neurons in the substantia nigra by 5-HT will probably decrease the normal function of the nigrostriatal pathway which is believed to be inhibitory on certain striatal neurons. Most available evidence indicates that DA is indeed inhibitory in the striatum. Iontophoretically applied DA inhibited striatal neurons (176,92) and electrical stimulation of the substantia nigra had an inhibitory effect, probably due to striatal release of DA, in the same way as iontophoretically applied DA (30). Degeneration of the nigrostriatal DA system caused an increased spontaneous firing frequency of certain striatal cells (120).

It could be assumed, therefore, that the behavioural excitation

that follows increased release of DA is due to an inhibition of certain striatal neurons receiving a DAergic input. The behavioural depression that follows a decrease in DAergic transmission may be due to a stimulation of the same or other neurons receiving a DAergic input from the substantia nigra. Although the mechanisms by which the striatal neurons influence motor behaviour are far from understood, the striatum seems to be an important centre of sensory-motor-integrating mechanisms and important link of the nigrostriatal pathway to the thalamus, the motor cortex and finally the spinal cord, all these being involved in the initiation of motor movement (383).

Lowering of the inhibitory nigral 5-HT influence, following selective asymmetric electrolesions of the DR nucleus, results, probably, to an indirect stimulation of the firing of the cell bodies giving rise to the nigrostriatal DAergic pathway, leading to an increased inhibition of the target neurons in the striatum. Following the above assumption, behavioural excitation should follow the increased release of DA and the resulting inhibition of the target neurons in the striatum. Similarly, drug-induced release of DA from the striatum (by amphetamine) should result in increased inhibition of these neurons and behavioural excitation.

Thus, the lesion in one side of the DR nucleus, which reduced selectively the 5-HT content of the ipsilateral substantia nigra, and the administration of amphetamine or apomorphine could be expected to be additive in their striatal effects; this might be manifested in the circling behaviour (spontaneous and drug-induced) towards the side of the lesion due to the biochemical deficit of some important neurons in the striatum ipsilateral to the lesion, compared to the contralateral side, i.e. because the increased activity of the nigrostriatal

DAergic pathway in the ipsilateral to the lesion side results in inhibition of the striatal neurons which seem to be an important and necessary link for induction of circling behaviour. The inhibitory nature of the nigrostriatal DAergic pathway seems, therefore, to be the reason why asymmetric lesions of the DR, which projects mainly to the substantia nigra, and asymmetric lesions of the MR, which projects to the striatum, resulted in spontaneous and apomorphine- or amphetamine-induced circling in opposite directions. However, other more elaborate interpretations of the circling behaviour should be considered, integrating several recent pharmacological findings.

5.4.4 Alternative interpretation of turning response to dopamine stimulants

It appears that an increase of DA turnover in the corpus striatum in the lesioned side is associated with an action of apomorphine and amphetamine on that side, which is greater than on the unoperated side and results in contralateral turning of MR-lesioned animals. However, the reverse is true of lesions affecting the substantia nigra (that is, DR lesions). Although DA turnover was increased in the side of the lesion in the substantia nigra, turning induced by apomorphine and amphetamine was in the ipsilateral direction. Several recent reports suggest a reciprocity between the effects of DA release at the two ends of the nigrostriatal system. Using a push-pull cannula technique, Niecullon et al. have shown that substantia nigra and corpus striatum react in opposite directions to sensory stimulation (384) and to local stimulation (385). From electrophysiological studies it has been suggested that DA release in the substantia nigra may inhibit the firing rate of DA cells (53,67). Thus, an increase in DA release in

the substantia nigra would be associated with a decrease in release in the corpus striatum. Biochemical studies in rats treated with haloperidol (Section 1) suggested the reciprocal effect is also true, that is: an increase in DA release in the corpus striatum is associated with a decrease in release in the substantia nigra.

Bearing these results in mind, it is less surprising that lesions which differentially affect one or the other of the ends of the nigrostriatal pathway would have opposite effects on behaviour. Increasing DA turnover in the substantia nigra specifically would be expected to reduce DA release in the ipsilateral corpus striatum and hence make the animal tend to turn in the opposite direction to one with the DA turnover raised in the corpus striatum. Thus, when stimulated with apomorphine or amphetamine, the observed turning behaviour was in opposite directions with lesions specific to MR or DR (Fig. 5.21). This concept could offer an interpretation of the behavioural excitation that follows raphe lesions or inhibition of 5-HT synthesis (319,320,321) or of the enhancement of the amphetamine-induced hyperactivity by these procedures (322,323,324). Furthermore, the apparent contradiction between the proposed inhibitory influence of 5-HT on DA release in the striatum and possibly the substantia nigra on one hand, and the production of the hyperactivity syndrome both by the DA releasing agent amphetamine and by the combination phenelzine plus tryptophan (which increases brain 5-HT) on the other, could be resolved by the demonstrated DA releasing properties of the latter, as described in Section 4.

Tryptophan loading of rats lesioned in the raphe nuclei, pre-treated with the MAO inhibitor phenelzine, induced turning that was not in the direction one would expect from a precursor of 5-HT, but in the

same direction as amphetamine and apomorphine (Fig. 5.21). This was surprising, in view of the known fact that this drug combination produces a marked increase of 5-HT concentration in brain. The amphetamine-like biochemical effects of this drug combination and especially the release of DA due probably to displacement by the newly synthesised 5-HT, as the results in Section 4 of the present Thesis indicate, would explain a similarity in the effect of the two treatments. The question why a small amount of released DA can predominate over the massively formed 5-HT in determining the direction of turning in both MR- and DR-lesioned animals cannot be answered from the present experiments. A first interpretation could be that more 5-HT accumulates in the intact side of the striatum or the substantia nigra (as it was found in the present study) and this produces greater inhibition of the DAergic neurons in this side, resulting in the DAergic asymmetry, an important step for the induction of turning. The direction of turning would then be towards the side with the striatal DAergic deficit (contralateral to the less inhibited striatal side), i.e. contralateral to the lesion in the MR and ipsilateral to the lesion in the DR-lesioned rats. A similar circling behaviour of rats with lesions of the nigrostriatal DAergic pathway was also observed following treatment with the combination phenelzine plus L-tryptophan and amphetamine: the former induced rotation in the same direction as the latter, i.e. ipsiversive (Section 4).

5.4.5 Relation of turning to stereotyped behaviour

In both types of lesion, amphetamine administration was followed by behavioural excitation and stereotyped behaviour, as described by Randrup and Munkvad (363). Rotation has been considered by some workers to be a facet of the amphetamine-induced stereotyped behaviour

(313). Evidence has suggested that the 5-HT innervation of the striatum does not influence the amphetamine-induced stereotyped behaviour (313). Randrup and Munkvad (272) also concluded that 5-HT receptors are not involved in amphetamine stereotyped behaviour. These considerations, however, would contradict the described behavioural effects of raphe lesions and the present experimental results. Accordingly, if the amphetamine-induced rotation in MR- and DR-lesioned animals is considered as part of the stereotyped behaviour produced by this drug, then the 5-HT receptors do not need to be directly involved in this behaviour. The direct effect of amphetamine on DA release, finally mediating the circling or stereotyped behaviour, could explain the lack of apparent involvement of 5-HT in amphetamine-induced stereotypy. This, however, does not exclude the possibility that alterations of the 5-HT input may affect behavioural syndromes mainly mediated by DA.

Obviously, the selectivity of the lesions is in no doubt, as it was histologically and biochemically demonstrated. The involvement of DA in the circling and other behavioural responses induced by amphetamine or apomorphine has clearly been demonstrated, whereas involvement of NA did not seem to be a factor contributing to the circling behaviour. Furthermore, obvious changes in 5-HT and 5-HIAA in the striatum or the substantia nigra correlated with the successful rotation after MR or DR lesions, respectively.

Additional evidence for involvement of 5-HT receptor stimulation in the circling behaviour was obtained from behavioural experiments. 5-Methoxy-N,N-dimethyltryptamine (5-MDT), a drug considered to be a specific central 5-HT receptor stimulant (336) produced in both types of lesion a dose-dependent rotation that was in opposite direction to

the rotation induced by apomorphine or amphetamine, i.e. ipsiversive in MR and contraversive in DR lesions (Fig. 5.21). The rotation was blocked by prior administration of methysergide, a 5-HT receptor antagonist (339). Thus, it is evident that the turning involves also 5-HT mechanisms which are distinct from the DAergic mechanisms and result in different behavioural responses. The finding that methysergide blocked only the turning and not the other behavioural effects of 5-MDT, could indicate that these effects are separated from the turning response or that they could not be inhibited by the doses of 0.5 or 1 $mg/_{kg}$ methysergide.

With regard to the circling behaviour in particular, the DA receptor agonists and the 5-HT agonist seemed to produce the opposite effect. When apomorphine and 5-MDT were administered simultaneously, no consistent rotation in either direction appeared in both MR and DR lesions. This finding provided further support for the proposed concept of tonic inhibitory control of the DAergic neurons by the serotonergic input to the substantia nigra and the corpus striatum. That this sequence is involved in the initiation of rotation is indicated by the finding that haloperidol, a DA receptor blocker, prevented the circling induced by the 5-HT receptor stimulant. By analogy, the 5-HT receptor blocker methysergide, presumably through temporary removal of the serotonergic inhibition of DAergic neurons (which is added to the surgical removal of this 5-HT influence from one side of the substantia nigra or the corpus striatum and together result in a bigger DAergic asymmetry in these areas) significantly speeded up the onset of the apomorphine effect and increased the number of turns during the first 5, the first 10 and during the total period of 45 min of the test (Fig. 5.17 and 5.18). Not only the circling, but also other

behavioural effects of apomorphine appeared as early as 30 sec after the i.p. administration of this drug, when it was given 45 min after methysergide.

The facilitation of the apomorphine effect on turning by methysergide is in close agreement with the reported enhancement of apomorphine-induced locomotor activity (388) and stereotypy (389) and also apomorphine-induced turning behaviour of rats with 6-hydroxydopamine lesions (390), although absence of serotonergic influence on apomorphine-induced stereotypy has been proposed by other workers (365). These findings also seem to be in agreement with the reported intensification of amphetamine-induced excitation by methysergide (364) or the reported enhancement of amphetamine locomotor action after surgical interruption of ascending serotonergic pathways (322,354) or after depletion of 5-HT from the brain by p-chlorophenylalanine (321); all this evidence points to a neuromodulatory influence of serotonergic neurons on the DAergic neurons in brain.

Further support for the concept of DAergic neurons finally mediating certain responses to various manipulations of the serotonergic system (such as the motor response) came from the pharmacological experiments of Green and Grahame-Smith (250); these investigators demonstrated that the hyperactivity syndrome (a behavioural response apparently dependent upon the release of 5-HT into the synaptic cleft) was blocked by α-methyl-p-tyrosine, a DA synthesis blocker.

The present study, however, suggests that methysergide does not intensify the amphetamine-induced rotation, as it does to the apomorphine effects, but it probably causes a reduction in the intensity of the rotation. Methysergide alone induced rotation in MR- but not in DR-lesioned rats, in the same direction as the DAergic stimulants, probably

due to an indirect effect that follows inhibition of 5-HT mechanisms. The demonstrated serotonergic asymmetry, which resulted from the lesion, seemed to persist or to be enhanced after 5-HT receptor blockade, at least with regard to the striatum. Simultaneous administration of 5-MDT and apomorphine resulted in no turning, whereas the amphetamine-induced turning was intensified by simultaneous injection of 5-MDT, in both MR and DR lesioned rats. The fact that the effect of apomorphine, a drug thought to stimulate postsynaptic DA receptors, was inhibited by 5-HT receptor stimulation probably at a presynaptic level of the DAergic neurons, tends to suggest that the circling responses to these drugs can be mediated by independent, different mechanisms.

The combination of amphetamine with the direct 5-HT receptor agonist increased the intensity of amphetamine-induced turning in both MR and DR-lesioned rats. Prior administration of methysergide decreased the effect of amphetamine in both types of lesion. These results contrast with the effects of apomorphine combined to a 5-HT agonist or antagonist. This could mean that a component of the amphetamine effect not common to that of apomorphine interacts with the 5-HT agonist and antagonist. This difference might be related to the presynaptic action of amphetamine (compared to the postsynaptic action of apomorphine), or to the 5-HT releasing properties of amphetamine (242) additional to its DA and NA releasing properties. The finding that 5-HT receptor stimulation significantly potentiates and 5-HT receptor blockade partially decreases the intensity of turning induced by amphetamine, implies that amphetamine has a direct or indirect stimulant effect on 5-HT receptors, contributing to the turning. This effect of amphetamine could be postulated to be on

the intact side of the striatum or the substantia nigra, since an effect on the 5-HT system in the lesioned side would induce turning opposite to the turning due to DA receptor stimulation; an action on the 5-HT release or reuptake processes would probably be more effective in the intact side than in the denervated side depleted of 5-HT. Therefore, when amphetamine alone is administered, its effects on the DAergic system predominate over the effects on the serotonergic system, causing turning in the same direction as apomorphine. Another possibility is that amphetamine is acting on the DAergic neuron at a presynaptic level to release DA, which in turn may be taken up by serotonergic neurons (244,245) to induce release of 5-HT which may serve as the inhibitory neuromodulator. If the target 5-HT receptors for the released 5-HT to exert its neuromodulatory effect (probably located on DAergic neurons) are blocked by methysergide, the amphetamine effect (mediated through the release of DA) will be more intense. Therefore, lesions of the serotonergic projection from one side of the DR or the MR leave the denervated afferent area deficient in 5-HT, or, perhaps, the 5-HT receptors 'supersensitive' and uninhibited by the dose of $0.5 mg/_{kg}$ of methysergide.

Thus, the 5-HT agonist and the antagonist have the effects on the response to amphetamine opposite to those on the response to apomorphine. Both 5-HT receptor stimulation and inhibition clearly affect the amphetamine-induced turning. Furthermore, if stereotyped behaviour depends on the integrity of the nigrostriatal DAergic pathway (8,382) and involves DA receptor stimulation (272), the fact that turning is induced by drugs acting specifically on 5-HT receptors opens other possibilities. The present data indicate that both DAergic and serotonergic processes are involved in the turning response, but the former may be the final

mediator of this response and the latter the modulator of its function, although the possibility of a direct 5-HT involvement in the initiation of this syndrome independently of the DAergic mechanisms should be considered.

If 5-HT blockade is, in one or the other way, reducing the effect of amphetamine on DA release and consequently on circling, several points may arise with reference to this concept. 5-HT released from its nerve terminals in brain, at physiological concentrations, acts probably on its postsynaptic receptors in the striatum and the substantia nigra to influence the DAergic neurons at a presynaptic level, by inhibiting the release of DA. 5-HT receptors could, therefore, be located on DA terminals. If circling is part of the stereotyped behaviour as has been suggested (313), the finding that 5-HT and DA receptor stimulation can induce rotation of DR- and MR-lesioned rats is in agreement with the demonstrated ability of both monoamines (5-HT and DA) to initiate a stereotyped response of rats when they are administered intrastriatally (386, 111). Furthermore, the effectiveness of intrastriatal DA application seems to be partially dependent upon an intact serotonergic innervation, since raphe lesions reduced the stereotyped behaviour induced by this treatment (368).

Therefore, the influence of 5-HT mechanisms upon stereotyped behaviour appears to be facilitatory, in contrast with the inhibitory nature of the serotonergic innervation of the striatum on locomotor activity (380, 321, 322, 313) and the lack of any effect on locomotor behaviour following lesions of the DR nucleus (380), which seems to innervate the substantia nigra (present study). Thus, the locomotor behaviour seems to be related to striatal mechanisms mainly, whereas stereotyped behaviour and circling behaviour seem to involve, to a

different degree, both striatal and nigral mechanisms. Considering the proposed relation of stereotyped and circling behaviour, the direction of circling might involve a mechanism independent of both, probably the nigrostriatal DAergic asymmetry.

5.4.6 Dopamine and 5-HT 'supersensitivity'

The rotation induced by both DA and 5-HT agonists needs further investigation. The contraversive rotation of MR-lesioned rats and the ipsiversive rotation of DR-lesioned rats following the administration of apomorphine or amphetamine seem to result from ipsilateral DA receptor stimulation. A state of 'supersensitivity' of the DA receptors could be postulated, similar to the denervation supersensitivity phenomenon observed after interruption of the nigrostriatal DAergic pathway (8,110). A 'supersensitive' response of the denervated DA-ergic neurons to direct or indirect stimulation of DA receptors is the possible necessary step in the initiation of turning. A direct striatal DA receptor stimulation by apomorphine and an indirect stimulation by amphetamine (through release of DA) or even by methysergide (through blockade of the remaining inhibitory 5-HT influence) may lead to the demonstrated contraversive turning of rats with asymmetric MR lesions. The preferential ipsilateral stimulation (causing the contraversive turning) may result from the abolition of the mainly originating from the MR nucleus inhibitory influence, and this might constitute the most important feature of the 'supersensitivity' phenomenon presumably occurring in MR-lesioned animals. A similar DA receptor 'super-sensitivity' could be postulated to occur in the substantia nigra. The reciprocity of the two ends of the nigrostriatal pathway with refer-ence to DA release may be the reason why the turning due to DR lesions is in opposite direction than the MR lesion-induced turning.

The fact that there is no consistent spontaneous turning in MR- or DR-lesioned rats, despite the increased DA release in the denervated side of striatum or substantia nigra, respectively, may indicate either that DA is inactivated before reaching its postsynaptic receptors or that continuous stimulation of these receptors by the released DA has caused decreased responsiveness to stimulation (subsensitivity), a phenomenon appearing when DA receptors in brain have been stimulated in a sustained manner (189). Subsensitivity of DA receptors, however, could not explain the apomorphine- and amphetamine-induced contralateral turning of MR-lesioned rats and the ipsilateral turning of DR-lesioned rats. It is, therefore, possible that DA is released and rapidly inactivated in the denervated side, thus leading to 'supersensitivity' of the postsynaptic DA receptors.

The fact that apomorphine and amphetamine induce rotation at higher doses and with lower maximum intensity than in the case of lesions of the nigrostriatal DAergic pathway (368,314) could be partly attributed to the limited extent of the serotonergic denervation; the remaining intact neurons are, probably, still functioning physiologically, i.e. they exert an inhibitory influence on DAergic neurons. Furthermore, the capability of DA to influence motor behaviour, by acting as a neurotransmitter, seems to surmount much higher concentrations of 5-HT, which could function either as a neurotransmitter or as a neuromodulator in connection with motor behaviour. This is suggested by the finding that the combination of MAO inhibition with tryptophan loading may be expressed behaviourally as an indirect DA receptor stimulant rather than a 5-HT receptor stimulant.

In addition to the hypothesised DA receptor 'supersensitivity' resulting from raphe lesions, the intense rotation induced by the 5-HT

receptor stimulant 5-MDT implies a distinct 5-HT postsynaptic receptor 'supersensitivity' mechanism functioning after denervation in both corpus striatum and substantia nigra. The idea of a preferential stimulation of the 5-HT receptors in the denervated side of the corpus striatum or the substantia nigra would be compatible with the finding of Bennett and Snyder (287) that raphe lesions did not reduce the 5-HT and LSD binding in forebrain membranes, but on the contrary increased it; the enhanced binding may reflect a depletion of endogenous 5-HT bound to postsynaptic receptors rather than synthesis of new 'supersensitive' binding sites. The 5-HT-sensitive adenylate cyclase, which seems to be associated with postsynaptic 5-HT receptors in brain (299,300) might also be involved in the mechanism of the 'supersensitive' response to 5-HT stimulation. The increased stimulation of the post-synaptic sites ipsilateral to the lesion, either in the substantia nigra or in the corpus striatum (probably located on DAergic cells or on DA-ergic nerve terminals), due probably, to the reduced competition by endogenous 5-HT, might result in an increased inhibition of DA release which reverses the effect of DA receptor stimulants, producing turning in the opposite direction (Fig. 5.21).

Chronic administration of neuroleptic drugs, which are known to block DAergic transmission, probably by receptor blockade, led to tolerance to the acute effects on striatal DA turnover (Section 3 of this Thesis). Administration of a DA receptor stimulant, such as apomorphine, after chronic neuroleptic treatment of normal rats, produced a 'supersensitive' response (148,80) whereas a single dose of the neuroleptic could prevent the behavioural and biochemical effects of DA receptor stimulation. Tolerance, and the 'super-sensitivity' stage that follows, have not yet been explained, and the

mechanisms underlying these phenomena are far from clear. A common feature of the 'neuroleptic-induced supersensitivity' and the 'denervation-supersensitivity', is the increased number of DA receptors in the striatum or in the denervated area (119,153) and the increased responsiveness of striatal neurons to DA receptor stimulation (120,80).

These phenomena, of the development of tolerance to the blockade of DAergic neurotransmission and of 'supersensitivity' to DA receptor stimulation, were demonstrated in the present study. Haloperidol given acutely blocked the turning induced by apomorphine or amphetamine in both MR and DR lesions, indicating the direct involvement of DAergic mechanisms in the circling behaviour induced by these drugs. Chronic administration of haloperidol, for 15 days, did not prevent the apomorphine- or amphetamine-induced turning, when these drugs were administered 1 hour or 24 hours after the last daily injection of haloperidol.

It seems from these observations that the hypothesised 'denervation-supersensitivity' of DA receptors, proposed as an important step in the initiation of turning in both MR and DR lesions, could be inhibited by an acute haloperidol injection but was unmasked after repeated administration of this neuroleptic. The 'drug-induced supersensitivity' of postsynaptic DA receptors was probably added to the 'denervation-supersensitivity', since the circling response of rats with MR lesions to apomorphine was significantly higher than the response to administration of apomorphine alone (without haloperidol pretreatment).

Rats with DR lesions also showed the phenomenon of tolerance to the neuroleptic drug, but their response to apomorphine was, probably, lower than the response to a single injection of apomorphine (without pretreatment with haloperidol). The difference in the effect of chronic

neuroleptic treatment on the MR- and the DR-lesioned animals cannot be readily explained. The finding of this study that the MR innervates the corpus striatum and the DR innervates the substantia nigra could probably provide an answer, in view of the different effects of chronic neuroleptic treatment on DA metabolism in the two areas reported in Section 3 of this Thesis; indeed, tolerance to the effect of haloperidol on the concentrations of HVA and DOPAC did not seem to develop in the substantia nigra on chronic treatment, but it did in the striatum.

Supersensitivity to amphetamine did not seem to develop in either MR or DR lesions following chronic neuroleptic administration. However, tolerance to the inhibitory effect of acute haloperidol on amphetamine-induced turning was observed after chronic treatment: the turning was of about the same intensity and in the same direction as after the administration of amphetamine alone (without haloperidol pretreatment), both 1 hour and 24 hours after the last injection of haloperidol. The same effect was noted in MR- and in DR-lesioned animals. The complexity of the biochemical effects of amphetamine (the 5-HT and DA releasing properties, in particular) could be the reason for the difference in this circling response compared to the effect of apomorphine. The release of 5-HT by amphetamine, which has not been implicated by any direct or indirect evidence in the tolerance to neuroleptic drugs, might counteract the 'supersensitivity' to the released DA in the denervated side. This DA 'supersensitivity' could be a reason for the grossly estimated 'supersensitive' behavioural response to amphetamine with regard to locomotor and stereotyped behaviour (5.3.4f), which seem to be far more complex processes than circling.

Although the precise interpretation of these findings is not possible from the present study, the close correlation between changes

in DA metabolism and circling (reported in this Section) give further support to the idea of a DAergic mechanism necessarily mediating the turning of rats with asymmetric MR or DR lesions.

Chronic administration of the 5-HT receptor blocker methysergide to rats with MR or DR lesions resulted in a phenomenon similar to the above. The inhibition of the turning induced by 5-MDT when methysergide was administered beforehand, was not seen on chronic administration of methysergide; this finding indicates that tolerance to the 5-HT receptor blockade had developed. The effect of the 5-HT receptor stimulant could not be blocked after 22-27 daily injections of methysergide; on the contrary, the intensity of turning was increased and could not be eliminated even with 2 or 4 times higher dose of the antagonist, given before the injection of the 5-HT agonist.

Thus, a specific raphe lesion-induced and a 5-HT receptor blockade-induced 'supersensitivity' could be postulated to exist, being additive in this rat-turning model, analogous to the same phenomena with reference to the DAergic system. The supersensitive response to 5-HT receptor stimulation after chronic blockade did not change the direction of turning and it did not prevent the imbalance produced by the lesion. On the contrary, the increased turning rate might imply an increase in the asymmetry between the two sides of the striatum or the substantia nigra, indicating probably a further increase in the sensitivity of the 5-HT receptors in the denervated side. Further studies involving estimations of the 5-HT sensitive adenylate cyclase and the number of 5-HT receptors in the denervated areas could provide more information about the effects induced by the lesion or the chronic methysergide treatment and elucidate the mechanism of 5-HT receptor 'supersensitivity'.

Available data on the acute effects of methysergide and 5-MDT on 5-HT metabolism indicate some similarities to the effects of neuroleptics and apomorphine on DA metabolism, respectively. Thus, administration of methysergide produced an increase in the turnover of 5-HT, reflected in an elevation of 5-HT and 5-HIAA in brain (339), probably due to activation of a positive feedback mechanism which increased the synthesis and utilisation of 5-HT. In contrast, stimulation of central 5-HT receptors by 5-MDT and by certain phenylethylamines (338), probably initiated a compensatory negative feedback mechanism which slowed down the turnover of 5-HT in brain. These regulatory processes, triggered by 5-HT receptors were still functioning in brain slices to control the release of labelled 5-HT induced by potassium (30mM) or by electrical stimulation (344,343). The similarity of these mechanisms with the regulatory mechanisms proposed for the DAergic neurons in brain (44) tends to support a generally similar process for the regulation of the biosynthesis and release of monoamines in the central nervous system.

5.4.7 5-HT as a neuromodulator

The DA 'autoreceptors' in the substantia nigra (53,67), probably mediating the effects of DA released from the dendrites (34) may constitute part of a possible mechanism of local modulation of DAergic cell activity. The demonstrated in vitro release of 5-HT (induced by potassium, 30mM) from slices of the substantia nigra (290) and the known ability of DA to enter serotonergic neurons (248) and displace under certain circumstances the endogenous 5-HT from its neurons (244), could possibly indicate an important neuromodulatory role of 5-HT in the substantia nigra, where it is known to be of inhibitory nature (64,315). The demonstrated axo-dendritic synapses of the serotonergic afferents

to the substantia nigra with other neurons of this area (73) offer the anatomical background for the neuronal interrelation of the serotonergic and the DAergic systems.

A similarity to the function of GABA in the substantia nigra, which is believed to be released by dendritically released DA (57), to be inhibitory on nigral cells (64) and to be stored in neurons originating outside of the substantia nigra (i.e. corpus striatum and globus pallidus, 68,82) is evident from the findings of the present study. The demonstrated ipsiversive circling behaviour of rats with unilateral lesions of the GABAergic striato-pallido-nigral pathway following the administration of apomorphine or amphetamine (45), also suggests that, perhaps, an additive or supplementary function could be postulated for the 5-HT and the GABA inputs to the substantia nigra in modulating the activity of the nigrostriatal DAergic pathway.

A presynaptic control of DA release in the striatum similar to that proposed for GABA could be postulated to exist, with 5-HT as the neuromodulator. The serotonergic afferent to the striatum could impinge on the DAergic nerve terminals at a preterminal level and modulate the release of DA, probably by acting on 5-HT receptors located on these terminals. Further experiments are needed in order to assess these possibilities and establish the anatomical site and the nature of the neuronal interactions of DA and 5-HT.

5.4.8 Conclusions

The asymmetric electrolytic lesions of the 5-HT-containing cell bodies in the rat midbrain, described in this Section, suggested that: 1) The median raphe nucleus appears to project unilaterally to the corpus striatum and not to the substantia nigra. The dorsal raphe nucleus appears to project unilaterally to the substantia nigra; a

minor serotonergic afferent to the corpus striatum may also originate from the dorsal raphe nucleus.

The lesions selectively damaged the 5-HT cells, without any damage to DA- or NA-containing neurons terminating in the striatum. 2) DA turnover was selectively increased in the terminal area where there was a reduction of 5-HT and 5-HIAA. These results indicate that the serotonergic projections to the corpus striatum and the substantia nigra have an inhibitory effect on DA metabolism in these areas.

3) The DA receptor agonist apomorphine and the DA releasing drug amphetamine induced turning in lesioned rats, which was contralateral to MR lesions and ipsilateral to DR lesions. The 5-HT receptor agonist 5-methoxy-N,N-dimethyltryptamine induced turning in opposite directions to the DA receptor agonists. The combination phenelzine plus L-tryptophan induced turning similar to that of DA receptor agonists. Participation of NA in the amphetamine-induced turning was excluded. 4) Blockade of 5-HT receptors by methysergide appeared to facilitate the circling response to apomorphine but rather reduced the amphetamine-induced turning. Conversely, stimulation of 5-HT receptors by 5-methoxy-N,N-dimethyltryptamine facilitated the amphetamine-induced turning and blocked the apomorphine-induced turning. Haloperidol blocked the response to 5-HT and to DA receptor agonists.

These results suggest that supersensitivity of the 5-HT receptors in the denervated area may develop after the lesions, but DA seems to be a necessary step in the initiation of turning. Apomorphine and amphetamine seem to induce turning by a different mechanism. Since the DA asymmetry does not seem to induce consistent, spontaneous turning, the response to DA receptor stimulation could be

ascribed to DA receptor supersensitivity on the denervated side.

5) The demonstrated rat turning model could be useful in differentiating drugs acting as agonists or antagonists of 5-HT or DA. It could also be used for the demonstration of tolerance to the effects of chronic blockade of 5-HT or DA neurotransmission and for the assessment of supersensitivity that follows.

6) The serotonergic projections to the corpus striatum and the substantia nigra may be part of alternative mechanisms regulating the activity of the nigrostriatal DAergic pathway by independently influencing its two ends, the cell bodies and the nerve terminals.

GENERAL DISCUSSION

Present work - Proposed model - Future work

Despite the wealth of various experimental procedures employed, limited and often inconsistent data were obtained relating to possible functional relationships between the DA-containing nigrostriatal pathway and other neuronal systems. Continued efforts to elucidate the nature of these interactions would seem crucial to any comprehensive view of sensory-motor integration and co-ordination of motor movement, in which the nigrostriatal pathway seems to be an important link, (383,394).

The work described in this Thesis suggests that several neuronal systems function in a very interactive manner. The nigro-striatal pathway appears to have contacts and interactions crucial to its function, both in the cell body area of the substantia nigra (Fig. 5.22) and in the terminal area of the corpus striatum (Fig. 5.23).

Several procedures were employed in an effort to elucidate certain interneuronal relationships which involve this pathway. Primary lesions of the DA cell bodies and treatments with drugs thought to act specifically on DA receptors provided evidence concerning the interaction of the DAergic with the cholinergic and the GABAergic systems. Selective increase of 5-HT by precursor loading or decrease by selective lesions of the raphe nuclei provided evidence for the DA - 5-HT interactions. On the basis of the proposed neuronal interactions, alternative interpretations of the action of drugs primarily affecting the DAergic system might be obtained. Finally,

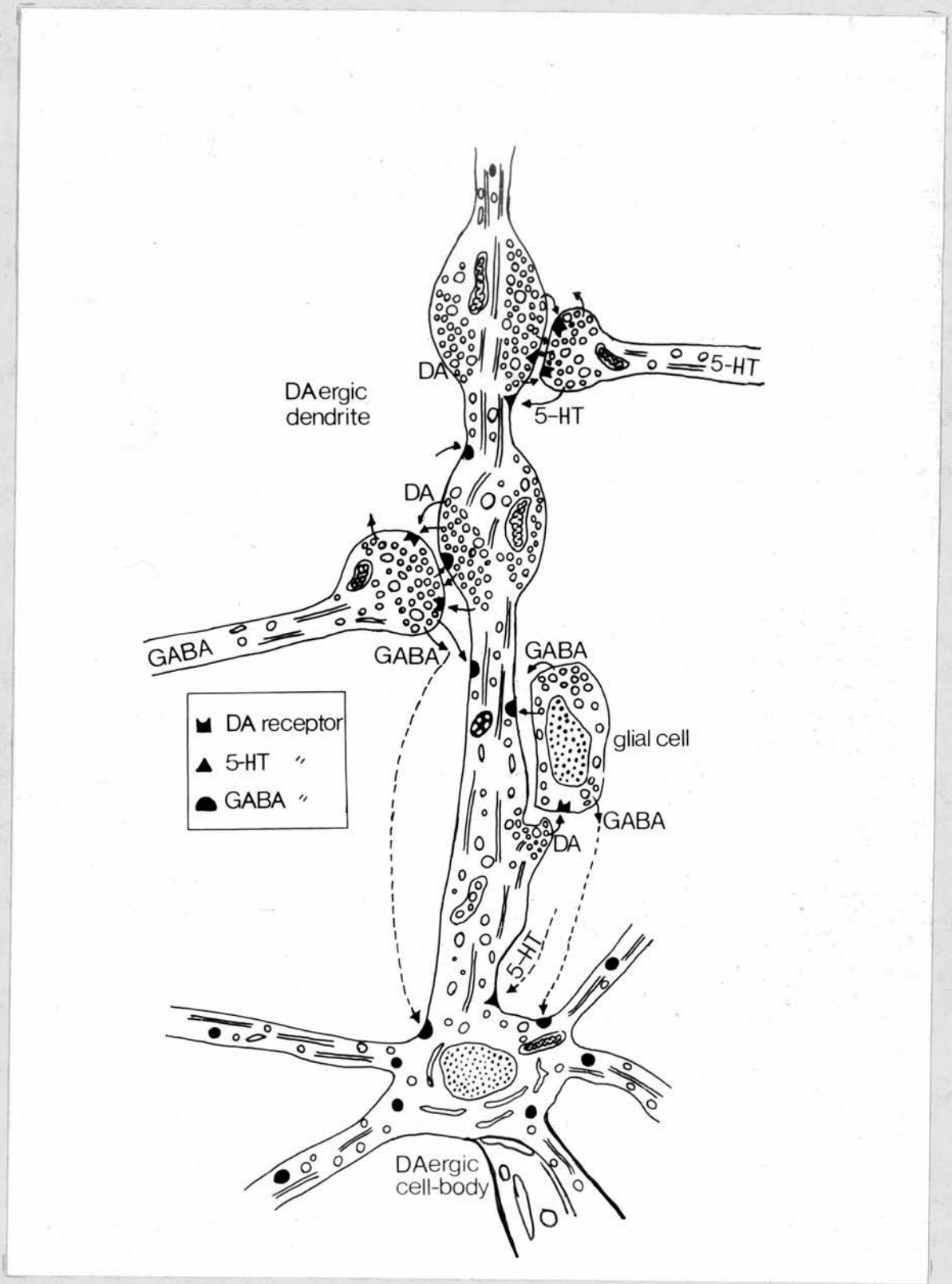


Fig. 5.22

Possible neuronal connections of a DAergic cell body and a dendrite in the substantia nigra

Arrows indicate the presumed direction of synaptic transmission, mediated through the respective receptors.

Possible neuronal connection with a glial cell, a 5-HT and a GABA-containing nerve terminal are depicted.

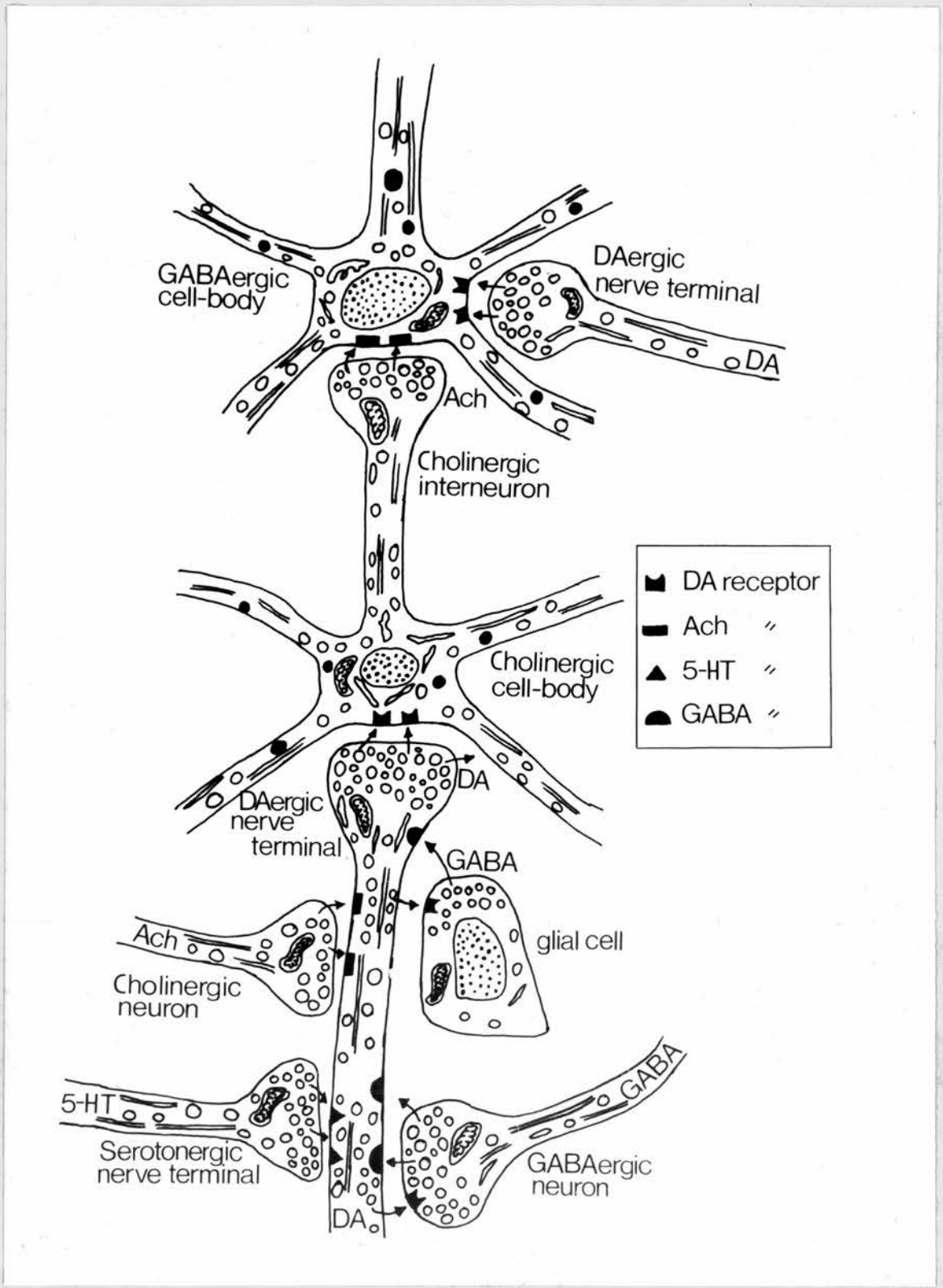


Fig. 5.23

Summary of possible neuronal connections in the corpus striatum

Arrows indicate the presumed direction of synaptic transmission mediated through the corresponding receptors.

The DAergic nerve terminal is depicted to have post-synaptic contacts with Ach- and GABA-containing cell bodies and presynaptic contacts with neurons containing Ach, GABA or 5-HT and glial cells containing GABA.

an attempt was made to formulate a comprehensive view of some neuronal influences on the nigrostriatal pathway.

The homeostatic mechanisms, such as hypersensitivity or tolerance of the DAergic system to certain experimentally-induced changes, may represent nothing more than a special case of a general biological phenomenon, that is, the capacity of the cell to compensate for changes (within limits) by adaptation of its activity (cellular adaptation). Even in these states, neuronal interactions might appear to be involved.

A more concrete basis of adaptation would be through feedback mechanisms. Such a mechanism for the control of the activity of the nigrostriatal pathway has been postulated to exist, with the striato-nigral GABAergic pathway as its morphological substrate (44,61). Several other hypotheses have been made based on the intranigral DA-DA interactions ('autoreceptor' theory, 53,34,33), the intranigral neuronal interactions (57,56), and the influence of the serotonergic input to the substantia nigra (87). The experiments reported in this Thesis were aimed at examining some aspects of these mechanisms.

A reciprocity of biochemical events related to DA neurotransmission was found between the area of the DA cell bodies and the nerve terminal area. Certain effects of haloperidol, the DA receptor blocker, and apomorphine, the DA receptor stimulant, on DA metabolism in the substantia nigra were contrary to those in the striatum (Section 1). Chronic haloperidol treatment also resulted in different effects on DA metabolism in the two areas (Section 3). Selective lesions of the 5-HT containing cell bodies in either the median or the dorsal raphe nuclei resulted in specific increases of DA metabolism in the corpus striatum or the substantia nigra, respectively, without any effect on

the other end of the nigrostriatal pathway (Section 5). Lesions of the DA cell bodies and consequent degeneration of nerve terminals in the striatum resulted in effects on cholinergic and GABAergic neurons which were dissimilar or were even in opposite directions in the two areas (Section 2). Some of these data could be taken as evidence against the existence of a GABA-containing striatonigral feedback pathway, since procedures affecting GABA in the striatum or the substantia nigra did not have effects on striatal DA metabolism consistent with such role. Strong evidence against the GABAergic neuronal feedback loop has resulted from other experiments (165,166). Similarly, the serotonergic projection from the DR to the substantia nigra appeared to be inhibitory on DA metabolism in this area, but it had no effect on DA metabolism in the striatum, suggesting that it is probably not the alternative feedback control of the nigrostriatal pathway.

The DA 'autoreceptor' theory (53,34) implied that DA released from the dendrites in the pars reticulata of the substantia nigra (33,34) acts on DA receptors located on the cell bodies or the dendrites to inhibit the cell firing rate. However, the finding that changes in the concentrations of DA metabolites (implying parallel changes in DA release) in the substantia nigra were not followed consistently by reverse effects on DA release in the striatum (Section 1) argues against this mechanism exclusively controlling the nigrostriatal pathway. Further argument against the theory of autoreceptor control comes from the reported lack of effect of 6-hydroxydopamine lesions, which destroyed the nigral DA neurons, on the DA-sensitive adenylate cyclase (probably associated with DA receptors) in the substantia nigra (18,56). On the contrary, location of this

adenylate cyclase in GABA- or substance P-containing nerve terminals, as suggested by other experiments (46) may indicate that these or other neurons are involved in the regulation of DA cell activity. Release of GABA by DA and by the DA releasing agent, amphetamine, has been demonstrated in the substantia nigra (57), where the inhibitory effect of GABA on DA cell firing is well documented (64, 82).

Feedback control by intranigral neuronal interactions could be mediated by some of the substances known to be present in this area of the brain. The finding that apomorphine and amphetamine appear to increase and haloperidol to decrease GABAergic activity in the substantia nigra (Section 3) might be in line with the proposed role of GABA as neuromodulator. Further evidence that DA may act as GABA mimetic, that is, to release GABA which in turn may inhibit the DA cells (Fig. 5.22) was obtained from the findings that lesions of the DA cell bodies resulted in reduction of GABA and GAD which could be reversed by DA receptor stimulation with apomorphine (Section 2). The latter finding is consistent with the hypothesised 'supersensitive' response of the DA receptors in the lesioned side (to apomorphine) and the resulting contralateral turning (8,110). It is also in agreement with the reported contralateral turning of rats following elevation of GABA in the substantia nigra (387) or ipsilateral turning following reduction of GABA in the same area (45). The chronic neuroleptic treatment offered additional evidence for this concept, by demonstrating a positive correlation between the changes in GABA or GAD and the concentrations of the metabolites of DA (Section 3). Although this finding contradicts the known inhibitory effect of GABA on DA cell activity, cellular adaptation to the effect of GABA might have resulted

from the chronic neuroleptic treatment.

In this context it is tempting to speculate that DA, by acting on its receptors located on other neuronal systems, may induce the release of inhibitory or excitatory neuromodulators of the DA cell activity. Thus, release of substance P or Ach, which have been shown to have excitatory effects in the substantia nigra (64,395), could constitute part of a local positive feedback control (Fig. 5.22). This has not been demonstrated yet. Further characterisation of this interrelation would require evidence of the existence of a DA-sensitive adenylate cyclase or DA receptors on nigral cholinergic neurons, (as has been shown for substance P) as well as cholinergic or substance P receptors on DA cells or dendrites. Furthermore, the ability of DA to release substance P and Ach and, conversely, the ability of Ach and substance P to release DA in the substantia nigra need to be demonstrated. The finding that oxotremorine, a cholinergic agonist, increases DA turnover in the substantia nigra (54) supports this idea. The reduction of the enzyme CAT after lesions of the substantia nigra (Section 2) also supports a 'trophic' influence of DA on nigral cholinergic neurons, in agreement with the proposed interrelation. The reported increase of striatal release of DA by substance P applied in the substantia nigra (396) provides further evidence for this hypothetical relationship.

The finding that 5-HT, released by serotonergic neurons originating in the raphe nucleus, may exert a tonic inhibition of DA cells (87), thus inhibiting DA release (Section 5) may be yet another influence on the nigrostriatal pathway, as depicted in the diagrammatic model of Fig. 5.22. 5-HT as well as GABA, could be released from their nerve terminals in the substantia nigra (probably in the

pars reticulata) to act as neuromodulators at some distance from the release sites, probably on the DA cell bodies in the pars compacta, or on the DA dendrites, thought to form synapses with these terminals (73). The release of these neuromodulators may, in turn, be controlled by dendritically released DA, as has already been suggested for GABA (57).

It is conceivable that a role for 5-HT similar to that of GABA could be demonstrated if DA and DA receptor agonists could release 5-HT in the substantia nigra, in a similar approach to that of Reubi et al. (57). Furthermore, a DA sensitive adenylate cyclase or DA receptors may be located on serotonergic terminals, similarly to the location of a DA-sensitive adenylate cyclase on GABAergic terminals (18,46), to mediate the release of 5-HT by DA. 5-HT (and GABA) receptor binding sites may be located on DA cells. A unilateral increase of 5-HT in the substantia nigra (e.g. by local application) may be expected to have a similar effect on motor behaviour (turning in particular) to the local application of GABA and GABA receptor agonists (387). A study of the effects of such a unilateral increase of 5-HT in the substantia nigra on the activity of the nigrostriatal pathway and on DA metabolism in the corpus striatum could test the hypothesis of Dray et al. (87) that the raphe projection to the substantia nigra may be the inhibitory-modulatory influence on the activity of the nigrostriatal pathway, as an alternative to the GABA-mediated control.

The finding that lesions of the DR nucleus resulted in an increase of DA metabolite concentrations in the substantia nigra but not in the striatum, replicates similar findings after lesions of the striatonigral GABA-containing pathways (45) and it is inconsistent with

the role assigned to the serotonergic input. The fact that neither of these lesions affected DA release from the striatum, but probably increased DA release in the substantia nigra may be suggestive of other possibilities: (a) 5-HT (and GABA) may act directly on DA dendrites to inhibit the release of DA in the substantia nigra and, therefore, may not have an effect on DA cell firing rate. (b) The release of DA may be regulated locally by serotonergic and GABAergic afferents to the substantia nigra and by serotonergic afferents and GABAergic axon collaterals impinging on DAergic neurons at a pre-terminal level in the corpus striatum. These mechanisms could be similar but independent at the two ends of the nigrostriatal pathway. (c) There might be a threshold of firing rate of the DA cells, below which there is no observable change in DA release at the nerve terminal. Therefore, reductions (even up to 50%) of the concentration of the neuromodulator in the substantia nigra may not affect the DA cell activity, because the 5-HT or GABA remaining after lesions of the respective neurons, may suffice to fulfill the role of neuro-modulator designated to them. (d) If GABA and 5-HT have additive or supplementary effects on the activity of the DA cells in the sub-stantia nigra, lesions affecting only the GABAergic or only the serotonergic input may not impair significantly this activity due to overfunctioning of the intact neuromodulatory neurons. The possibility that both inputs act together to control the activity of the nigro-striatal pathway could be assessed by procedures (such as lesions) that involve both GABA and 5-HT afferents to the substantia nigra. (e) In view of the proposed existence of two or more compartments for 5-HT (240), of which the small 'functional' pool rather than the large 'storage' pool determines the functional state of the serotonergic

neurons, procedures which would prevent replenishment of the functional pool would indicate more precisely the role of 5-HT in relation to the DA cell activity. Similarly, the functional significance of alterations in the concentration of GABA needs to be clarified before any conclusions can be drawn from experimentally-induced changes in its total concentration in the substantia nigra. The occurrence of high amounts of GABA in the glial cells (207) as well as in the GABA nerve terminals may suggest that procedures reducing only neuronal GABA are not necessarily valid methods for the assessment of its functional significance in relation to the DA cell bodies. GABA, of glial origin, may participate in the regulation of DA cell activity, as speculated in Fig. 5.22

Further work is also needed for the elucidation of the nature and the location of the proposed DA 'autoreceptor' in the substantia nigra, the possible existence of more than one DA receptor in this structure, and the possible dissociation of DA-sensitive adenylate cyclase from DA receptors. These points would clarify the postulated DA-DA interactions, and the functional significance of the dendro-dendritic synapses and the dendritic release and uptake of DA.

The diversity and often the reciprocity of changes induced experimentally in the corpus striatum and the substantia nigra concerning DA metabolism tend to suggest that local regulatory mechanisms may be functioning independently in both areas. However, the nature of the interactions in the striatum may be similar to those postulated for the substantia nigra. Although reciprocity of effects induced experimentally on DA release at the two ends of the nigrostriatal pathway, without involvement of an intermediate feed-back pathway would contradict the generally accepted view that changes

in cell activity and impulse flow are followed by similar changes in transmitter synthesis and release from the nerve terminals (25, 26,27), the proposed models (Fig. 5.22 and 5.23) may offer viable alternatives.

According to the proposed scheme of neuronal interrelations in the striatum (Fig. 5.23) the DAergic neurons originating in the substantia nigra have both pre- and post-synaptic contacts with other neurotransmitter systems. Although the functional substrate for these contacts has been formulated, morphological evidence is still required and remains an interesting subject for future work.

On the basis of in vitro experiments, presynaptic control of DA release in the striatum by excitatory cholinergic (174) and by inhibitory GABAergic neurons (192) impinging at a preterminal level on DA nerve terminals have been proposed. A reduction of GABA receptor binding sites in the striatum following lesions of the DA cells in the substantia nigra with 6-hydroxydopamine, has been demonstrated (196), whereas evidence concerning the cholinergic (muscarinic or nicotinic) receptors after such lesions is needed in order to establish the existence of this neuronal interrelationship.

Evidence obtained from the lesion experiments reported in Section 5, suggests that 5-HT released from serotonergic terminals originating in the MR may have an inhibitory effect on DA release, probably at a preterminal level. Although the inhibitory nature of this projection on behavioural phenomena that in all probability involve striatal DA receptor stimulation appears to have strongly documented support (present study, 322,388,250), its presynaptic contact with the DAergic nerve terminals awaits further morphological and biochemical evidence. Primarily, location of postsynaptic 5-HT receptors (or

5-HT sensitive adenylate cyclase, thought to be associated with them, 299,287) on DA neurons has to be demonstrated, to form the basis of the 5-HT-DA neuronal interactions. It is premature, however, to speculate on the relative importance of the nigral and the striatal serotonergic inputs (which the present study demonstrates to have distinct origin), despite the finding that selective lesions of these pathways resulted in reciprocal biochemical and behavioural effects (Section 5). The ability of accumulated 5-HT to enter DA and NA containing neurons and displace these monoamines as observed after tryptophan loading (Section 4), does not appear in physiological states, and probably represents a different interrelation, not mediated through receptors.

The functional importance of the presynaptic contacts of the various neurons thought to impinge on DA nerve terminals as neuro-modulators could be elucidated if a link could be shown between the activity of these neurons and the activity of DAergic nerve terminals, i.e. DA synthesis or release in the striatum. With reference to 5-HT, there is no such concrete evidence. The ability of DA to release 5-HT at low concentrations in vitro (397), may suggest that the abundantly released DA from the striatal nerve terminals is capable of inducing in turn the release of 5-HT from serotonergic nerve terminals. Alternatively, the reported finding of a projection from the caudate nucleus to the area of the median raphe in the cat (328) may be hypothesised to form the basis of a feedback loop, an attractive possibility awaiting experimental evidence. The existance of such feedback loops related to the serotonergic system has already been proposed (332), even implicating the habenula as an intermediate nucleus feeding back to the raphe cells information from the 5-HT nerve terminals

(317,327,331).

The postsynaptic contacts of the DAergic nerve terminals appear to be less obscure. Postsynaptic DA receptors (and DA-sensitive adenylate cyclase) seem to be located in Ach and GABA-containing cells in the striatum (94,95) and to mediate respectively a direct inhibition (Sections 2 and 3, also 90,91,93) and, probably, a stimulation (Sections 2 and 3). In addition, an indirect contact of DAergic with GABAergic neurons, through short cholinergic interneurons has been proposed (106,105) and is also supported by data reported in Section 3. Thus 'trophic' influences of DAergic neurons seem to exist on cholinergic or GABAergic neurons, in parallel, or in consecutive arrangement, as deduced from the results of this Thesis and the work of others. The parallel existence of a DA-Ach-GABA and a DA-GABA synaptic link, of which one would be inactivated during development of tolerance to continuous exposure to a drug (such as haloperidol, Section 3) may be an example of the general 'redundancy theory' of neuronal adaptation (398). The excitatory indirect influence of DA on GABA-containing cells (through short cholinergic interneurons, Fig. 5.23) may be deactivated after prolonged blockade of DA neurotransmission by haloperidol.

The GABA- and Ach-containing striatal cells seem to be the origin of the neurons impinging on DAergic nerve terminals (174,192). It might be speculated that these neurons form part of a local feedback mechanism, activated through stimulation of DA receptors located on their cell bodies. Such an effect would be predicted, on the basis of the finding that activation of the striatal cholinergic system is associated with an increased release of DA (increase of the metabolite concentrations, Sections 2 and 3), the reverse, probably being also true. Similarly increases or decreases of GABAergic activity in the striatum generally correlate with reciprocal changes in DA release, as

acute and chronic haloperidol treatment has indicated (Sections 3). The reported increase of DA receptor binding sites in the striatum after degeneration of DAergic neurons (119) and after chronic neuroleptic treatment (153) may also be in agreement with this scheme. Definite conclusions cannot be drawn, however, until this concept has been further elucidated.

In a manner seemingly incompatible with the above scheme, lesions of the DA cell bodies and the resulting 'supersensitivity' of post-synaptic DA receptors to stimulation by apomorphine did not result in a 'supersensitive' change in the DA metabolite concentrations. An alternative explanation would be that apomorphine acts on presynaptic DA receptors (on intact nerve terminals) to exert an influence on DA synthesis and release, as suggested by other investigators (63, 163). The ability of haloperidol to induce an increase in the striatal DA turnover, even in a 90% degenerated DAergic system, supports a predominantly postsynaptic effect which is probably mediated through a local feedback mechanism. These neuronal interactions may help to explain the hyperactivity of DAergic neurons remaining intact after lesions, a phenomenon appearing to represent an 'intrinsic' DA neuronal adaptation mechanism. Use of other procedures is needed, however, in order to establish a conceptual framework of these 'feedback' mechanisms.

APPENDIX

A SENSITIVE GAS LIQUID CHROMATOGRAPHIC ASSAY FOR HOMOVANILLIC ACID (HVA) AND 3,4-DIHYDROXYPHENYLACETIC ACID (DOPAC) IN BRAIN TISSUE

INTRODUCTION

In order to study the regional distribution of the metabolites of DA in discrete areas of the rat brain and the effect of various treatments on their concentration, it was found necessary to have a reliable and sensitive assay procedure. The development of gas liquid chromatographic (glc) methods for the estimation of 4-hydroxy-3-methoxyphenyl acetic acid (homovanillic acid, HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC) was an important advance in the study of DA metabolism in brain. The greater sensitivity and specificity of these methods, compared to the existing fluorimetric techniques made them much more useful, especially in the study of changes in the concentrations of these metabolites in small, discrete regions of the brain of small laboratory animals, such as rats and mice, and enabled assays to be made without pooling tissues from several animals.

The method used in the present study is a modification of the method of Pearson and Sharman (41) with considerable improvements and is based on the formation of the hexafluoro-isopropyl esters of the trifluoroacetyl derivatives of HVA and DOPAC and their estimation by glc. Such a technique was first described by Dziedzic et al. (391) and was applied to the estimation of HVA in human urine and CSF. Their method involved the extraction of the HVA into ethyl acetate, evaporation of the organic solvent and the formation of the hexafluoroisopropyl (HFIP) derivative of trifluoroacetylated (TFA) HVA which could be reliably measured at submicrogram amounts by glc fitted

with an Electron Capture Detector (ECD). The presumed reaction (391) is illustrated in Fig. A1.

Pearson and Sharman (41) extended the method to include the estimation of DOPAC in the same sample of brain tissue or CSF. Their method, slightly modified, was used in some of the experiments described in this Thesis (Section 4).

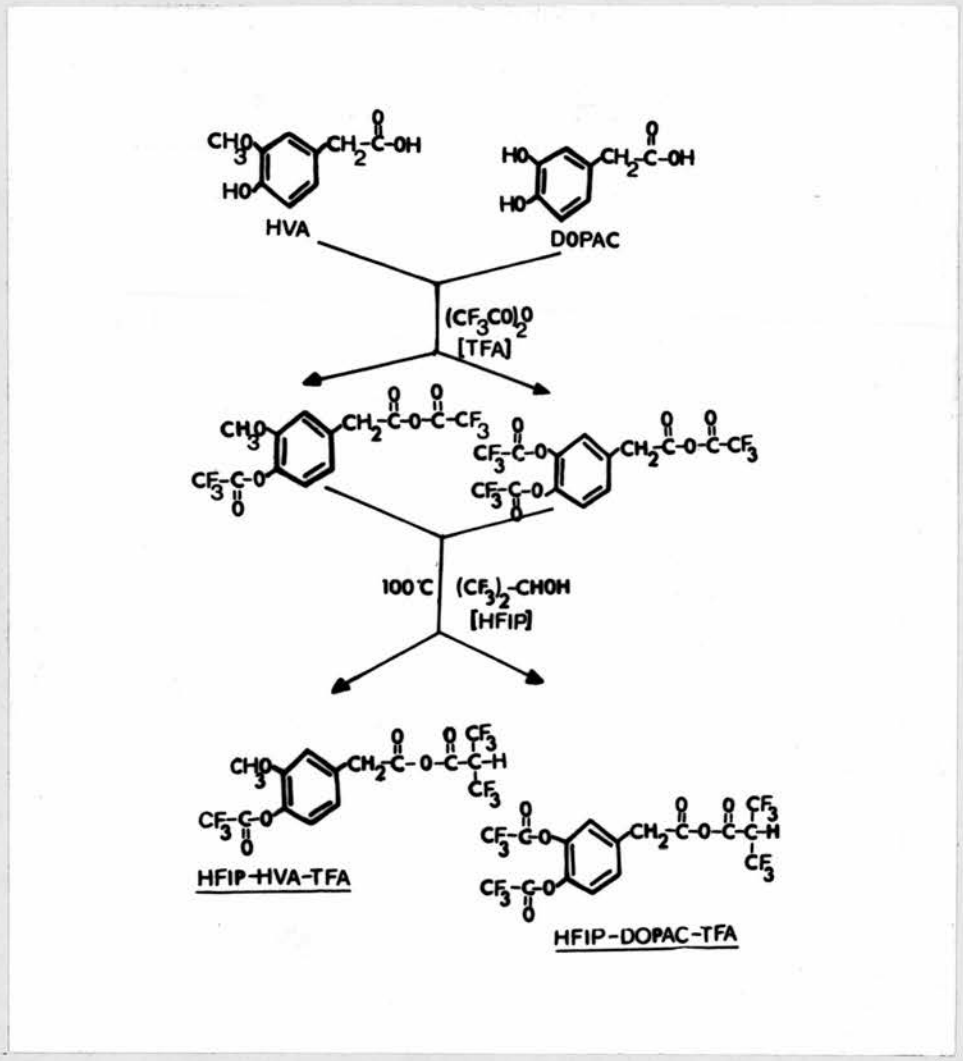


Fig. A.1

The presumed reaction for the formation of the trifluoracetyl-hexa-fluoroisopropyl (TFA-HFIP) derivatives of HVA and DOPAC

A.1 Examination of the method of Pearson and Sharman (41)

A.1.1 METHODOLOGY

The assay procedure was as follows:

Homogenisation and removal of protein from tissue sample. Brain tissue (up to 200mg) was dissected out, weighed and placed on dry ice. It was then homogenised in an all glass homogeniser (Jencons 5ml model) with 1ml 0.1M HCl, and the pestle was finally washed with another 1ml 0.1M HCl which was added to the homogenate. Perchloric acid (25 µl of 72%) was added to the homogenate and mixed thoroughly by vortex-mixing for 5 sec. The homogenate was then transferred to a 15ml centrifuge tube with washing with 2 x 1ml 0.1M HCl, approximately 100mg KCl (Analar) were added and the tube contents vortex-mixed for 15 sec. After centrifugation for 15 min at 3,000 revs/min, the supernatant was transferred into a 5ml cellulose nitrate tube containing a saturating amount of solid KCl and mixed well by suction into and expulsion from a teat pipette. The mixture was frozen in liquid nitrogen, and allowed to thaw sufficiently for the formation of a layer of water between the ice and the walls of the tube, before centrifuging at 10,000 revs/min for 15 min at 10°C, using a high speed head.

Extraction of HVA and DOPAC. The supernatant after centrifugation was transferred to a centrifuge tube and more solid KCl was added (about 100mg) with mixing. Ice-cold ethyl acetate (CT grade, 4ml) was added and the two layers vortex-mixed for 1 min. After centrifugation at

3,000 revs/min for 3 min, 3.5ml of the ethyl acetate layer was transferred to another test tube. Another 2ml of ethyl acetate was added to the first tube, vortex-mixed and centrifuged as before. Two ml of the ethyl acetate layer was removed and combined with the 3.5ml of the first extraction. The combined ethyl acetate extracts were evaporated under a jet of dry oxygen-free nitrogen at 55°C until the volume was reduced to about 0.2ml. The evaporated extract was transferred to a reaction vial (1ml 'Reacti' vial, Pierce Chem. Co.) with washing with 0.3ml of ethyl acetate. The solution in the vial was evaporated to dryness under a jet of oxygen-free dry nitrogen at room temperature.

Derivatisation of HVA and DOPAC. To the residue in the reaction vial 0.2ml of trifluoracetic anhydride (TFA, Aldrich Chem. Co.) and 0.1ml of 5% boron trifluoride etherate (BDH) in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP,BDH) were added and mixed. The vial was then tightly closed with a screw-on cap with a teflon liner and heated for 1 hour at 100°C in a heating block. After cooling to room temperature, the vial was opened and the contents evaporated under a gentle jet of oxygen-free nitrogen to give an oily residue. The residue was dissolved in 1.0ml CT grade ethyl acetate containing 100ng/ml pentafluorophenylbenzoate (PFPB). The PFPB served as an internal standard for the subsequent glc. The HVA and DOPAC derivatives were 'separated and estimated by glc.

Gas liquid chromatography. The solution of the derivatives (1 or 2 µl) was chromatographed under the following conditions:

Column	2% SE52 on Diatomite CQ in 9ft glass column (4mm i.d.)
Carrier gas	5% methane - 95% argon
Flow rate	at least 50ml/min (pressure 40 p.s.i.)
Injection port temperature	230°C
Column temperature	115°C
ECD temperature	200°C

A.1.2 RESULTS

The recoveries of DOPAC and HVA (25-50 ng) added to homogenates of rat brain tissue and estimated by this procedure were 60 ± 8% (n = 12) and 58 ± 10% (n = 9), respectively. The retention times of TFA - HFIP derivatives of HVA and DOPAC relative to the internal standard were 0.52 and 0.35 respectively, as reported by Pearson and Sharman (41).

A.1.3 DISCUSSION

Although the method theoretically offers great advantages over the previously used fluorimetric or gas chromatographic methods, such as increased sensitivity and specificity, it proved to be unreliable for routine assays. Also, the preparatory work needed (redistillations of reagents and solvents) made the procedure even lengthier. In extracts from brain tissue, unidentified peaks were often observed overlapping to a greater or lesser extent the peaks of HVA or DOPAC, thus decreasing the specificity. The reproducibility was also unsatisfactory, probably because of the fact that the procedure was complicated and involved too many stages at which variable losses could occur. The stage at which insertion of the supernatant into liquid nitrogen in cellulose nitrate tubes was done was not satisfactory, because breakages

of tubes with loss of frozen samples were very frequent. The extraction into ethyl acetate and then the transfer of the reduced volume of ethyl acetate into the reaction vial was time consuming. The recoveries of both HVA and DOPAC were low and the estimation of these metabolites in tissues not very rich in DA would not be possible unless tissues from several animals were pooled together. The 5% boron trifluoride (BF_3) etherate in HFIP, used with TFA for the derivatisation of HVA and DOPAC was found to give a chromatogram with many unidentified peaks. Very often the DOPAC peak was covered by an unknown peak which disappeared when BF_3 was omitted. The loss of HVA derivative, thought to form a complex of high b.p. with BF_3, was prevented when evaporation under nitrogen was carefully performed until an oily residue remained in the vial.

A.2 Detailed procedure for the estimation of HVA and DOPAC in small portions of brain tissue

A.2.1 INTRODUCTION

The need to improve the method so as to increase its sensitivity and reproducibility and decrease its complexity led to the introduction of a number of modifications. The whole procedure was scaled down. Small plastic centrifuge tubes (Eppendorf) of 1.5ml were used throughout in the modified method, with smaller volumes being used both for homogenisation and for extraction with organic solvents. Extraction manipulations were carried out in 1.5ml Eppendorf tubes with snap closures. These modifications enabled a larger number of samples to be worked up simultaneously. Though the basic procedure of extraction of the acidic metabolites from acidified supernatant into ethyl acetate and subsequent derivatisation was retained, certain stages were found to be unnecessary or even to cause additional problems rather than aid. It was found that the introduction of an extraction of the homogenate with toluene before extraction of the acids with ethyl acetate proved useful. The additional toluene extraction step was found not to affect the recovery of HVA and DOPAC, but removed much material interfering with the assay, so that 'cleaner' chromatograms were obtained. In some of the later experiments, additional purification of the samples was obtained by freezing the aqueous extract, after the toluene extraction, in liquid nitrogen. Centrifugation after thawing gave separation of a clear supernatant from a small amount of precipitate.

Three extractions into ethyl acetate were carried out and the extracts combined in a reaction vial and evaporated to dryness. This step increased the recovery of the method. BF_3 etherate was omitted from the reaction mixture to give a 'cleaner' chromatogram. One or two toluene extractions could be carried out, depending on how clean the supernatant was after the first extraction. Usually, one extraction and subsequent insertion of the tube into liquid nitrogen was used.

A.2.2 METHODOLOGY

The improved method finally employed for the estimation of HVA and DOPAC in brain tissue is detailed below:

Homogenisation of brain tissue and preliminary purification. Rat brain tissue of known weight not exceeding 100mg and previously stored in liquid nitrogen was thawed and transferred into a 1.5ml centrifuge tube with a plastic snap-closure (Eppendorf tube). Ice-cold perchlorid acid, 200 µl 0.4M, was added and the tissue was homogenised for 1 min with an electrically driven (6000r.p.m.) teflon tipped pestle. Another 600 µl of cold 0.4 M perchloric acid was added, the tube contents were mixed and centrifuged for 4 min at room temperature at 14,000 revs/min in a bench (Eppendorf) centrifuge. The supernatant was transferred into an Eppendorf tube and a saturating amount of powdered KCl was added. The contents were vortex-mixed for 15 sec and centrifuged for 4 min at 14,000 revs/min. The clear supernatant, transferred to another Eppendorf tube, was extracted with 500 µl of ice-cold toluene by vortex-mixing for 30 sec and separating by centrifugation for 4 min at 14,000 revs/min. The organic layer was aspirated off and discarded. If a thick layer of lipid remained on the surface of the aqueous phase, then either the extraction with 500 µl toluene was repeated or the tube

contents were kept frozen in liquid nitrogen for 30 min or longer, thawed and centrifuged, a clear supernatant then being separated from the precipitated lipids.

Extraction of HVA and DOPAC. The HVA and DOPAC were extracted from the aqueous layer into 0.5ml of ice-cold ethyl acetate CT grade Reeve-Angels) by vortex-mixing for 1 min. The layers were separated by centrifugation for 4 min at 14,000 revs/min. The organic layer was transferred into a 1 ml reaction vial ('Reacti vial', Pierce Chem. Co.), care being taken not to transfer any of the aqueous phase, since this might prevent the trifluoroacetylation reaction that follows. The extractions with 0.5 ml ethyl acetate were repeated twice more, the extracts being combined in the reaction vial and then evaporated to dryness under a stream of nitrogen at room temperature.

Derivatisation of the extracted HVA and DOPAC. To the residue in the vial, 0.2 ml of redistilled TFA (Gold Label, Aldrich) and 0.1 ml of redistilled HFIP ('special for spectroscopy', BDH) were added (from ampoules kept sealed since the distillation) and the reaction vial closed tightly with a screw-cap fitted with teflon liners. The vial contents were vortex-mixed and heated for 1 hour at 100°C in a thermostatically controlled heating block. After it had cooled to room temperature, the vial was opened and the contents evaporated under a gentle stream of nitrogen at room temperature to remove the excess reagents. Evaporation was continued until a brown or yellow oily residue remained for the glc.

Gas liquid chromatography. This residue in the vial was dissolved in 1 ml of redistilled ethyl acetate, CT grade, containing 100 ng of PFPB which served as the internal standard. The contents of the vial were

vortex-mixed and pipetted into a 2 ml hypovial which was closed with a rubber cap held secure with an aluminium seal. The hypovial was placed in the rack of an automatic injection system attached to a Hewlett-Packard glc. About 2 µl were injected automatically with a 701N Hamilton microsyringe, usually every 60 min. The injection volume was constant, with ± 1% repeatable accuracy.

Conditions of gas liquid chromatography. A Hewlett-Packard 5700 series glc fitted with an auto-sampler and a 15 mCi ^{63}Ni ECD was employed. Operating conditions were the same as in the original method described above.

Recoveries of HVA and DOPAC through the procedure. The recovery of the acids through the method was determined as follows: Homogenates were divided into two equal portions. One portion, to which known amounts of HVA and DOPAC (usually 25, 50, 100 ng) were added, was carried through the procedure in parallel with the other portion of homogenate. The peak areas of the HVA and DOPAC in the homogenate with the standards minus the peak areas of these metabolites in the homogenate without the standards represent the net HVA and DOPAC recovered through the procedure. The recoveries were then calculated by comparison with the peak areas obtained from standards of HVA and DOPAC dissolved in ethyl acetate, which were added to reaction vials (usually the same amounts as above, i.e. 25, 50, 100 ng), evaporated to dryness and derivatised.

Calculations. The peak area method (393) was used for the claculation of the metabolite content of every sample. After triangulation of the peak, the area was taken as the height x half the base of the triangle. Standard curves were constructed from a series of HVA and DOPAC (10 - 100 ng) dissolved in ethyl acetate, which had been

evaporated to dryness under nitrogen, incubated for 1 hour at 100°C with TFA and HFIP and then blown down under nitrogen, dissolved in 1 ml of ethyl acetate containing 100 ng/ml PFPB and injected into the glc. A series of standards was injected in every assay. The peak areas of these standards were calculated as well as the peak area of the internal standard. A standard curve was constructed as ratio of HVA or DOPAC derivative peak area to the peak area of PFPB against ng of HVA or DOPAC present in the sample. The relation was linear over a wide range of HVA and DOPAC tested, i.e. 5-200 ng. Best-fit straight lines for graphs, evaluated by the method of least squares (using an Olivetti computer programme), were always used for the calculation of the amount of HVA and DOPAC in the unknown samples. A typical chromatogram of brain tissue sample is shown in Fig. A2.

Preparation of pentafluorophenylbenzoate (PFPB) used as internal standard for the glc. This substance was prepared by a method described by Pearson and Sharman (41). Pentafluorophenol (BDH) was redistilled under reduced pressure (b.p. 264°C at about 15 mm Hg, water pump). The pentafluorephenol (1 g) was dissolved in approximately 15 ml of 10% NaOH, the glass stoppered tube being kept ice-cold. Excess benzoyl chloride (BDH) (about 2 ml) was added and the mixture shaken for 10 min during which time an oily precipitate appeared. This precipitate was extracted into 10 ml dichloromethane. The dichloromethane extract was then dried over anhydrous sodium sulphate and the solvent removed by evaporating under a stream of nitrogen. The residue of PFPB was dissolved with warming in a minimum of redistilled hexane, the solution rapidly filtered and cooled to 0°C. The crystals formed were pure PFPB. After they were dried overnight at room temperature, until no smell of benzoyl chloride remained, they were stored in a

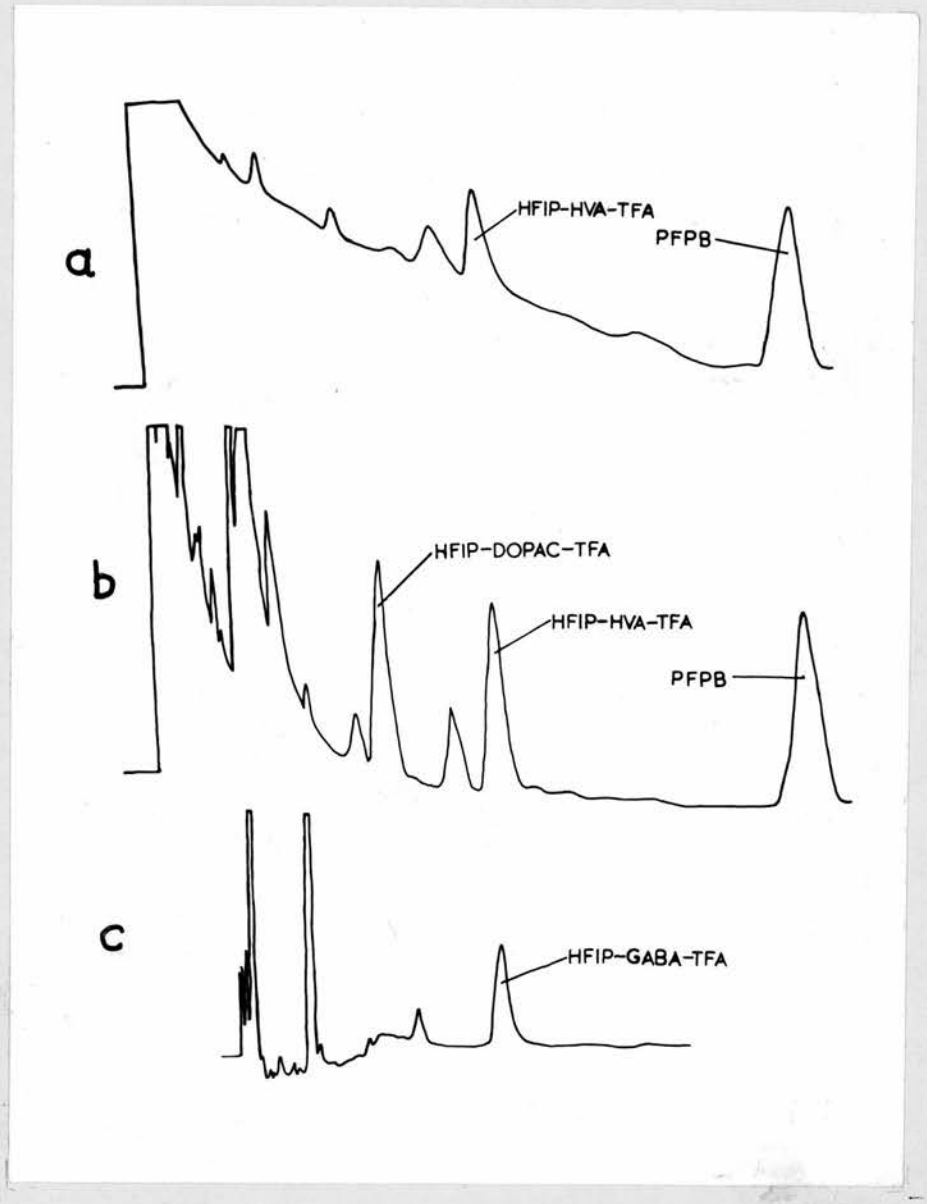


Fig. A.2

Chromatograms of (a) 0.5 ml of cerebrospinal fluid, (HVA), (b) brain tissue (HVA, DOPAC) and (c) brain tissue (GABA). The peaks corresponding to the trifluoroacetyl-hexafluoroisopropyl (TFA-HFIP) derivatives and to the internal standard pentafluorophenyl benzoate (PFPB) are indicated. The column was 9 ft long packed with 2% SE52 coated on Diatomite CQ. Oven temperature was 115°C for (a) and (b) and 92°C for (c). Detector temperature was 230°C. Carrier gas was argon with 5% methane, at flow rate 60 ml/min (pressure 40 p.s.i.)

desiccator. Solutions containing 100 $ng/_{ml}$ were prepared in ethyl acetate as required for use as an internal standard.

Distillation of reagents. The reagents for the derivatisation of HVA and DOPAC were redistilled at atmospheric pressure. TFA boiled at 39.5°C, HFIP at 57-58°C. Ethyl acetate (b.p. 77-78°C) for the final injection into the glc was redistilled and was also the hexane (BDH) that was used for the preparation of PFPB. Small fractionation columns (Quick-fit) were used for all the redistillations.

Column for glc-choice of packing material. In most of the experiments the glc column was packed with a methyl silicone gum (SE52) coated on a diatomaceous earth support (Diatomite CQ or Chromosorb Q). A 2% loading of the liquid phase SE52 on the solid support of Diatomite CQ 100-200 mesh was used. Several liquid phases coated on Diatomite CQ were tested and the relative retention times of the HVA and DOPAC derivatives are given in Table A1. The stationary liquid phase SE52 and a similar non-polar silicone gum liquid phase, SE30, with 3% loading were tried, as was a polar fluorinated silicone, QF1, with 3% loading. The solid support, coated with the liquid phase, was purchased from Hewlett-Packard (SE52), Pye-Unicam (SE30) and Analabs (QF1). The linearity of the response and the sensitivity of the fluorinated derivatives were also the same, so either of the SE52 or SE30 stationary phases could be used effectively. On the other hand, the QF1 due to its polarity tended to retain the polar HVA and DOPAC derivatives in preference to the less polar PFPB, so that the relative retention times were 1.29 and 1.50, respectively. Though this liquid phase offered the advantage of giving HVA and DOPAC derivative peaks at a safe distance from most of the unidentified peaks that could interfere, it had low efficiency and resolution, because the two peaks

were wide and often overlapping, compared to the sharp and separated peaks obtained when the SE52 and SE30 were used. A problem of inconsistent linearity of the responses was another reason for discarding the QF1 liquid phase. As can be seen in Table A1, the relative retention times for the 2% SE52 and the 3% SE30 liquid phase coating were about the same.

Amounts of the derivatives corresponding to as little as 1 ng of DOPAC or HVA could be detected using the above assay and glc conditions, provided the column packing was of a high enough quality to provide good efficiency and resolution.

The column was filled with the packing material according to the standard simple technique: The coated support was added in small amounts into the column from the free end, while at the other end a water aspirator was connected to the column which was plugged with glass-wool to assist the packing. An electric vibrator was used to speed up the settling of the packing material into the column.

Conditioning of the column. Conditioning is the process used to remove from the raw column absorbed air and also impurities in the liquid phase, residual solvent from the coating operation and other undesirable impurities and to make it ready for analytical use. A special no-flow conditioning procedure was used for the above silicone gum liquid phase columns and it was carried out only once for a new column. The column was installed in the instrument with the detector end unconnected and it was heated at 100°C for 30 min with a normal flow rate of carrier gas. The carrier gas flow was stopped and the column was heated at 310°C for 90 min. The column was afterwards allowed to cool to about 100°C, the carrier gas was turned on again and the regular conditioning was continued for about 16 hours at 120°C, after which

the detector end was connected again. The column was then ready for use. Regular conditioning with normal flow rate and at a temperature higher than the one used in the routine assays (usually 150°C) was performed in order to remove accumulated trash from the column.

Modified method for cerebrospinal fluid (CSF) and amniotic fluid (AF). The method described above for the estimation of HVA and DOPAC in brain tissues was found suitable, with some slight modifications, for the determination of these metabolites in CSF and AF from human subjects. The final version of the method used for the CSF assays is as follows: Ice-cold CSF (0.5ml) was mixed with 0.4 ml of 1M HCl in a 1.5 ml plastic centrifuge tube (Eppendorf) with attached snap closure. A few crystals of KCl were added and the mixture vortex-mixed for 30 sec. After centrifugation for 4 min at 14,000 revs/min., the clear supernatant was transferred into another Eppendorf tube, 0.5 ml ice-cold toluene was added, and the assay continued as described for brain tissue samples.

The recoveries of 25-100 ng of HVA and DOPAC added in the CSF and carried through the procedure were 76 ± 11% (n = 18) and 75 ± 10% (n = 13) respectively. A peak with relative retention time 0.35 was wrongly taken as DOPAC in some samples, contrary to many reports that DOPAC exists in subnanogram amounts per 1 ml of CSF, which cannot be measured by this technique. After careful assessment this peak was ignored as non-specific. A typical CSF chromatogram is shown in Fig. A2.

The same procedure was applied to AF (0.2 - 0.5 ml), but with the additional step of precipitation of proteins with 25 µl of concentrated perchloric acid (72% $w/_v$). Values obtained from human AF were ($ng/_{ml}$):

HVA	DOPAC
115	57
146	53
109	40

Recoveries of standards were 60 ± 9% (n = 7) for HVA and 59 ± 4% (n = 7) for DOPAC.

Incorporation of GABA assay in the glc procedure. In parallel with the assay of the DA metabolites, the assay of the amino acid GABA by glc could also be carried out on a portion of the same brain extract. After precipitation of proteins, 25 or 50 µl aliquots were taken from the clear supernatant and assayed as described in Section 2. Since GABA is a relatively stable compound, the aliquots could be safely kept at -20°C until it was convenient to perform the assays. A typical GABA chromatogram is shown in Fig. A2.

Attempts to identify unknown peaks of the chromatogram. Various attempts were made to identify some of the unknown peaks appearing frequently on chromatographic tracings. It was of particular interest to discover whether any of the other acidic metabolites of catecholamines or even of 5-HT were among these peaks. The TFA-HFIP derivatives of potential metabolites were prepared from the pure substances. Known amounts of such standards were dissolved in ethyl acetate and a suitable volume evaporated to dryness in a 'Reacti vial' under a stream of nitrogen at room temperature. The compound was reacted with 0.2 ml TFA and 0.1 ml HFIP at 100°C for 1 hour in a metal heating block. After evaporation of the excess reagents, the oily residue was dissolved in ethyl acetate and portions injected into the gas chromatograph.

Vanillyl mandelic acid (VMA), a minor metabolite of NA in the rat brain (21) gave a derivative showing a sharp peak, with the peak area increasing linearly up to 200 ng VMA on the SE30 and SE52 liquid phase columns. Like HVA and DOPAC, the limit of detection was about 1 ng VMA. The relative retention time (with PFPB as internal standard) was 0.425, so it would appear on a chromatogram between the peaks of

the HVA and the DOPAC derivative. No such peak was detected in brain samples, indicating that the concentration of VMA in brain tissues is below the limit of sensitivity of this method and in consequence is very low. This is in agreement with the findings of other workers (226,21).

The estimation of 5-HIAA was attempted by the same procedure, but no peak could be obtained, either in brain tissues rich in this metabolite or in pure standards added in reaction vials, probably because the b.p. of the TFA-HFIP derivative of 5-HIAA is much higher than 115°C.

The compound p-hydroxyphenylacetic acid, a potential metabolite of p-hydroxyphenylethylamine (tyramine) was another acidic agent forming fluorinated derivatives with low b.p. Standards of this phenolic acid gave linearly increasing peak sixes when reached in the same way as the HVA and DOPAC. The standard curve was linear up to 200 ng with a retention time of 0.198, relative to PFPB. Standards added to brain extracts were recovered at the end of the procedure, but brain extracts failed to give consistently any peak with this retention time, indicating that this compound does not exist in the rat brain in amounts measurable by this method.

The derivatisation of the phenolic compounds m-hydroxyphenylacetic acid, 3,4-dihydroxymandelic acid and dihydroxyphenylethyl alcohol was attempted, but no peak could be consistently obtained at column temperature 115°C, after they were reacted for 1 hour at 100°C with 0.2 ml TFA and 0.1 ml HFIP.

A.2.3 RESULTS AND DISCUSSION

Recoveries. The modified method showed remarkably higher recoveries from brain tissues than did the Pearson-Sharman method. For HVA it was 97 ± 7% (n = 9) compared to 58 ± 10% (n = 9) and for DOPAC it

was 93 ± 10% (n = 7) compared to 60 ± 8% (n = 12) with the original method. Since the recoveries were almost complete, quoted estimates of HVA and DOPAC in tissues have not been corrected for the small loss (unless otherwise stated).

Retention times. The glc conditions employed in this method produced satisfactory separation of the peaks corresponding to HVA and DOPAC, both from each other and from other unidentified peaks (Fig. A2). The relative retention times under operating conditions (2% SE52, column temp. 115°C, flow rate 50 ml/min) were 0.52 for HVA and 0.35 for DOPAC, the PFPB appearing at about 40 min. There was no detectable variation in the relative retention times under the constant glc conditions and, therefore, during the routine assays, the peaks appearing at these retention times were assumed to be due to the derivatives of HVA and DOPAC without any further test of specificity. The available means of testing the specificity of the method were randomly applied, as will be described below. When interfering unidentified peaks made the recognition of the HVA and DOPAC peaks difficult, the sample was run under different glc conditions and it was discarded if that was not successful. There was no peak interfering with the internal standard in any of the experiments.

Precautions during the assay procedure.

1. There are reports of post-mortem rises of DA metabolites, especially DOPAC (392); therefore, dissection of the tissues should be rapid and the tissues frozen in liquid nitrogen as quickly as possible. For the analyses the thawed tissue should be homogenised without delay, with care being taken to keep the temperature as low as possible. The homogenates and the subsequent brain extracts should be kept on ice throughout the procedure.

2. For a quantitative extraction of the acidic metabolites into ethyl acetate with no loss into the prior toluene extracts, the pH of the supernatant should be between 1 and 2. With amounts of tissue greater than 100 mg, the pH of the supernatant may be insufficiently low.

3. The extraction with ethyl acetate should not be proceeded unless a clear aqueous solution is obtained, with no layer of lipids on the surface. If a second toluene extraction is not effective in removing such a lipid layer, then freezing in liquid nitrogen and subsequent centrifugation after partial thawing may be effective. Finally, careful aspiration of the lipid layer will provide a clear aqueous solution.

4. Cold ethyl acetate must be used for the extractions. As much of the organic layer as possible is transferred into the reaction vial, but usually it was found not advisable to try to take off more than 0.4 ml, since any trace of water in the reaction vial would prevent the trifluoroacetylation by inactivating the TFA.

5. The screw-cap vials must be tightly closed for the reaction. A fresh teflon liner should be used each time to ensure a complete seal. The reaction should take place in a fume-cupboard because the reagents are corrosive and poisonous. After the incubation the vials must be left for about 10 min to cool down to room temperature before the evaporation under nitrogen is started. A gentle jet of nitrogen should be used, to avoid evaporation of the TFA-HFIP derivatives of HVA and DOPAC (which occurs at relatively low temperatures). Complete evaporation gives no consistent recoveries.

6. The derivatives, dissolved in ethyl acetate containing the internal standard, must be injected into the glc apparatus not later than 48 hours after they have been prepared. The closed vials containing these solutions for injection must be kept in a refrigerator until use.

Assessment of specificity of the glc procedure. Various ways of assessing the specificity of the glc method were used. The observation that a few unidentified peaks appeared regularly on the chromatogram with relative retention times in close proximity to those of HVA and DOPAC derivatives raised the question of possible interference of other unknown peaks with the peaks of HVA and DOPAC derivatives. Overlapping of peaks is very common in glc and this possibility was examined. Since the standard and most effective way of identifying and analysing unknown peaks, that is mass fragmentography (combined glc-mass spectrometry), was not available, other techniques were used.

The first approach was through a simple decrease of the operating column temperature of 115° to 110° or 105°C. This procedure, which could separate overlapping peaks, did not change the appearance of the HVA or DOPAC peaks. Single symmetrical peaks were obtained, indicating that they represent a single HVA or DOPAC derivative, as assumed from the fact that the relative retention times of both remained constant at temperatures 105°, 110°C and 115°C. In practice, this procedure was the most reliable way of answering questions of specificity in routine assays of brain tissues. When a similar problem arose and persisted for relatively long time and in more tissue samples, further steps were taken in order to separate overlapping peaks (if any) and ensure the specificity of the assay.

First, several columns of different length and different packing material were used. A column with a polar liquid phase, such as QF1, may separate two overlapping peaks representing derivatives of different polarity. As shown in Table A1, the HVA and DOPAC derivatives, which are highly polar compounds (Fig. A1), were retained strongly by the polar liquid phase, and their absolute retention times were higher than that of PFPB, as one would expect because of their difference in

fluoro-groups and hence the difference in polarity. Again, examination of brain samples with a 3ft column loaded with 3% QF1 on Chromosorb Q did not reveal any peak consistently accompanying and interfering with the peaks of HVA or DOPAC derivatives. The relative retention times for the HVA and DOPAC derivatives were 1.29 and 1.50, respectively. Other columns with a silicone gum liquid phase, such as OV1 and SE30 were used, and in all cases there was no indication of non-specificity of the peaks corresponding to HVA and DOPAC. The relative retention times of the two derivaties, obtained using columns of different length or liquid phase and also for different column temperatures are shown in Table A1.

Second, the specificity of the peaks appearing on chromatograms from brain samples was tested by a different approach, a pharmacological treatment. A group of 3 rats was injected with a large dose of the MAO inhibitor phenelzine sulphate (75 $mg/_{kg}$ i.p.) and killed 2 hours later. The HVA and DOPAC were determined in the corpus striatum of these rats in parallel with tissue from 3 rats treated with saline. Since MAO activity is a necessary step in the metabolism of DA leading to formation of HVA and DOPAC, its inhibition should lead to a marked reduction or even dissappearance of the two metabolites from the brain. Indeed, in the glc tracing there was no apparent peak, either in the position of the HVA or of the DOPAC derivative and hence no detectable levels of these metabolites were present in the striata of the phenelzine-treated animals. Extracts from the saline-treated controls showed sharp peaks, with relative retention times of 0.52 - that of the HVA derivative - and of 0.35 - that of the DOPAC derivative. It should be borne in mind that if an interfering peak represented an acidic metabolite of some unidentified monoamine, this should have also dis-

Table A.1

The retention times of the trifluoroacetyl-hexafluoro-isopropyl (TFA-HFIP) derivatives of HVA and DOPAC

Column length and liquid phase			Retention time HVA	Retention time DOPAC	Column temperature (°C)
9 ft	SE 52	2%	0.52	0.35	115
9 ft	SE 30	3%	0.55	0.37	115
3 ft and 9 ft	QF 1	3%	1.29	1.50	115
3 ft	OV 1	3%	0.525	0.37	115
3 ft	SE 30	3%	0.53	0.35	110
3 ft	OV 1	3%	0.53	0.37	110

The retention times (RT) of the HVA and DOPAC derivatives relative to that of the internal standard (pentafluorophenyl benzoate, PFPB) were estimated using columns with various packing materials and at different column temperatures

$$RT = \frac{\text{retention time of HVA (or DOPAC) derivative}}{\text{retention time of PFPB}}$$

The carrier gas was argon containing 5% methane with a flow rate of 60 ml/min (pressure of 40 p.s.i.) The detector temperature was 230°C.

appeared after MAO inhibition. Various ways of causing increase in DA metabolism were also used in subsequent studies, and selective effects on HVA and DOPAC were demonstrated. The regional distribution of the metabolites using the glc method was in agreement with other studies, providing additional evidence for the specificity of the method.

Third, an additional stage of purification was added to the method. That was an extraction of HVA and DOPAC from the ethyl acetate extracts into borate buffer pH 8.6, adjustment of the pH of the buffer to about 1 with conc. HCl and finally back extraction into ethyl acetate and evaporation to dryness, as described above. This tedious procedure did not produce any change in the chromatogram related to the size or shape of the peaks corresponding to HVA and DOPAC.

All the ways presented above of testing the specificity of the definitive version of the assay procedure led to the conclusion that there was no substance consistently present in brain tissue interfering with the HVA or DOPAC assay and, therefore, the peaks with relative retention times 0.52 and 0.35 were due only to the HVA and the DOPAC derivative, respectively. In conclusion, the evidence that the definitive version of the method was specific, sensitive, uncomplicated but reliable and allowed for the assay of as many as 36 samples in one day (up to the stage of injection in the glc), was a satisfactory indication that one could use this technique to study DA metabolism is discrete, small areas of the rat brain with accuracy and reproducibility.

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